

## CRYSTALLIZATION OF IGF-1

### Background of the Invention

#### Related Applications

5 This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60/267,977 filed February 9, 2001, and provisional application no. 60/287,072 filed April 27, 2001, the contents of which are incorporated herein by reference.

#### Field of the Invention

10 This invention is directed to a crystalline form of human insulin-like growth factor-1 (IGF-1) and more particularly to a crystal of human IGF-1, a method of crystallization thereof, and its structure, obtained by x-ray diffraction. In addition, the invention relates to methods of identifying new IGF-1 agonist molecules based on biophysical and biochemical data suggesting that a single detergent molecule that contacts residues known to be important for IGF-1 binding protein (IGFBP) interactions binds to IGF-1 specifically, and blocks binding of IGFBP-1 and IGFBP-3.

#### Description of Related Disclosures

15 There is a large body of literature on the actions and activities of IGFs (IGF-1, IGF-2, and IGF variants). Human IGF-1 is a serum protein of 70 amino acids and 7649 daltons with a pI of 8.4 (Rinderknecht and Humbel, Proc. Natl. Acad. Sci. USA, 73: 2365 (1976); Rinderknecht and Humbel, J. Biol. Chem., 253: 2769 (1978)) belonging to a family of somatomedins with insulin-like and mitogenic biological activities that modulate the action of growth hormone (GH) (Van Wyk *et al.*, Recent Prog. Horm. Res., 30: 259 (1974); Binoux, Ann. Endocrinol., 41: 157 (1980); Clemmons and Van Wyk, Handbook Exp. Pharmacol., 57: 161 (1981); Baxter, Adv. Clin. Chem., 25: 49 (1986); U.S. Pat. No. 4,988,675; WO 91/03253; WO 93/23071). IGFs share a high sequence identity with insulin, being about 49% identical thereto. Unlike insulin, however, which is synthesized as a precursor protein containing a 33-amino-acid segment known as the C-peptide (which is excised to yield a covalently linked dimer of the remaining A and B chains), IGFs are single polypeptides (see Figure 1).

25 In the developing embryo, the absence of IGF-1 leads to severe growth retardation that continues post-natally (Baker *et al.*, Cell, 75: 73-82 (1993); Powell-Braxton *et al.*, Genes Dev., 7: 2609-2617 (1993); Liu *et al.*, Cell, 75: 59-72 (1993); Liu *et al.*, Molecular Endocrinol., 12: 1452-1462 (1998)). While most (greater than 75%) of serum IGF-1 is produced by the liver in response to growth hormone, this liver-derived IGF-1 has been shown to be unnecessary for post-natal body growth in mice (Sjogren *et al.*, Proc. Natl. Acad. Sci. USA, 96: 7088-7092 (1999)). Rather, it is the locally produced, non-hepatic IGF-1, acting in a paracrine/autocrine manner, which appears to be responsible for most of the post-natal growth-promoting effects of IGF-1 (Schlechter *et al.*, Proc. Natl. Acad. Sci. USA, 83: 7932-7934 (1986); Isaksson *et al.*, Science, 216: 1237-1239 (1982)). Consistent with its growth-promoting effects, IGF-1 is a powerful mitogen, regulating diverse cellular functions such as cell-cycle progression, apoptosis, and cellular differentiation (reviewed in Jones and Clemmons, Endocr. Rev., 16: 3-34 (1995) and in LeRoith, Endocrinology, 141: 1287-1288 (2000)).

35 IGFs have been implicated in a variety of cellular functions and disease processes, including cell cycle progression, proliferation, differentiation, and insulin-like effects in insulin-resistant diabetes. Thus,

IGF has been suggested as a therapeutic tool in a variety of diseases and injuries (for review, see Lowe, Scientific American (March/April 1996), p. 62). Due to this range of activities, IGF-1 has been tested in mammals for such widely disparate uses as wound healing, treatment of kidney disorders, treatment of diabetes, reversal of whole-body catabolic states such as AIDS-related wasting, treatment of heart conditions such as congestive heart failure, and treatment of neurological disorders (Guler *et al.*, Proc. Natl. Acad. Sci. USA, 85: 4889-4893 (1988); Schalch *et al.*, J. Clin. Metab., 77: 1563-1568 (1993); Froesch *et al.*, Horm. Res., 42: 66-71 (1994); Vlachopapadopoulou *et al.*, J. Clin. Endo. Metab., 12: 3715-3723 (1995); Saad *et al.*, Diabetologia, 37: Abstract 40 (1994); Schoenle *et al.*, Diabetologia, 34: 675-679 (1991); Morrow *et al.*, Diabetes, 42 (Suppl.): 269 (1993) (abstract); Kuzuya *et al.*, Diabetes, 42: 696-705 (1993); Schalch *et al.*, "Short-term metabolic effects of recombinant human insulin-like growth factor I (rhIGF-I) in type II diabetes mellitus", in: Spencer EM, ed., Modern Concepts of Insulin-like Growth Factors (New York: Elsevier: 1991) pp. 705-715; Zenobi *et al.*, J. Clin. Invest., 90: 2234-2241 (1993); Elahi *et al.*, "Hemodynamic and metabolic responses to human insulin-like growth factor-1 (IGF-I) in men," in: Modern Concepts of Insulin-Like Growth Factors, Spencer, EM, ed. (Elsevier: New York, 1991), pp. 219-224; Quinn *et al.*, New Engl. J. Med., 323: 1425-1426 (1990); Schalch *et al.*, "Short-term metabolic effects of recombinant human insulin-like growth factor 1 (rhIGF-I) in type II diabetes mellitus," in: Modern Concepts of Insulin-Like Growth Factors, Spencer, EM, ed., (Elsevier: New York, 1991), pp. 705-714; Schoenle *et al.*, Diabetologia, 34: 675-679 (1991); Usala *et al.*, N. Eng. J. Med., 327: 853-857 (1992); Lieberman *et al.*, J. Clin. Endo. Metab., 75: 30-36 (1992); Zenobi *et al.*, J. Clin. Invest., 90: 2234-2241 (1992); Zenobi *et al.*, J. Clin. Invest., 89: 1908-1913 (1992); Kerr *et al.*, J. Clin. Invest., 91: 141-147 (1993); Jabri *et al.*, Diabetes, 43: 369-374 (1994); Duerr *et al.*, J. Clin. Invest., 95: 619-627 (1995); Bondy, Ann Intern. Med., 120: 593-601 (1994); Hammerman and Miller, Am. J. Physiol., 265: F1-F14 (1993); Hammerman and Miller, J. Am. Soc. Nephrol., 5: 1-11 (1994); and Barinaga *et al.*, Science, 264: 772-774 (1994)).

The patent literature also abounds with disclosures of various uses of IGF-1, or compounds that increase active concentration of IGF-1, to treat mammals, especially human patients, for example, U.S. Pat. Nos. 5,714,460; 5,273,961; 5,466,670; 5,126,324; 5,187,151; 5,202,119; 5,374,620; 5,106,832; 4,988,675; 5,106,832; 5,068,224; 5,093,317; 5,569,648; and 4,876,242; WO 92/11865; WO 96/01124; WO 91/03253; WO 93/25219; WO 93/08826; and WO 94/16722.

The IGF system is also composed of membrane-bound receptors for IGF-1, IGF-2, and insulin. The Type 1 IGF receptor (IGF-1R) is closely related to the insulin receptor in structure and shares some of its signaling pathways (Jones and Clemmons, *supra*). The IGF-2 receptor is a clearance receptor that appears not to transmit an intracellular signal (Jones and Clemmons, *supra*). Since IGF-1 and IGF-2 bind to IGF-1R with a much higher affinity than to the insulin receptor (Cascieri *et al.*, Biochemistry, 27: 3229-3233 (1988)), it is most likely that most of the effects of IGF-1 and IGF-2 are mediated by IGF-1R (Humbel, Eur. J Biochem., 190:445-462 (1990); Ballard *et al.*, "Does IGF-I ever act through the insulin receptor?", in Baxter *et al.* (Eds.), The Insulin-Like Growth Factors and Their Regulatory Proteins, (Amsterdam: Elsevier, 1994), pp. 131-138).

IGF-1R is an  $\alpha 2\beta 2$  heterotetramer of disulfide-linked  $\alpha$  and  $\beta$  subunits.  $\alpha\beta$  dimers are themselves disulfide linked on the cell surface to form a covalent heterotetramer. As in the insulin/insulin receptor

complex, IGF-1 binds to the IGF-1R with a 1:2 stoichiometry (De Meyts, *Diabetologia*, 37: S135-S148 (1994)), with a high affinity site ( $K_d$  about 0.4 nM) and a low affinity site ( $K_d$  about 6 nM) (Tollefsen and Thompson, *J. Biol. Chem.*, 263: 16267-16273 (1988)). The x-ray crystal structure of the first three domains of IGF-1R has been determined (Garrett *et al.*, *Nature*, 394, 395-399 (1998)). It contains three distinct domains (L1, Cys-rich, L2). Mutations that affect IGF-1 binding map to the concave surface of the receptor.

IGF-1R is a key factor in normal cell growth and development (Isaksson *et al.*, *Endocrine Reviews*, 8: 426-438 (1987); Daughaday and Rotwein, *Endocrine Rev.*, 10:68-91 (1989)). Increasing evidence suggests, however, that IGF-1R signaling also plays a critical role in growth of tumor cells, cell transformation, and tumorigenesis (Baserga, *Cancer Res.*, 55:249-252 (1995)). Key examples include loss of metastatic phenotype of murine carcinoma cells by treatment with antisense RNA to the IGF-1R (Long *et al.*, *Cancer Res.*, 55:1006-1009 (1995)) and the *in vitro* inhibition of human melanoma cell motility (Stracke *et al.*, *J Biol. Chem.*, 264:21554-21559 (1989)) and of human breast cancer cell growth by the addition of IGF-1R antibodies (Rohlik *et al.*, *Biochem. Biophys. Res. Commun.*, 149:276- 281 (1987)).

The IGFs are potent breast cancer cell mitogens based on the observation that IGF-1 enhanced breast cancer cell proliferation *in vitro* (Cullen *et al.*, *Cancer Res.*, 50:48-53 (1990)). Breast cancers express IGF-2 and IGF-1R, providing all the required effectors for an autocrine-loop-based proliferation paradigm (Quinn *et al.*, *J Biol. Chem.*, 271:11477-11483 (1996); Steller *et al.*, *Cancer Res.*, 56:1761-1765 (1996)). Because breast cancer is a common malignancy affecting approximately one in every eight women and is a leading cause of death from cancer in North American women (LeRoith *et al.*, *Ann. Int. Med.*, 122:54-59 (1995)), new rational therapies are required for intervention. IGF-1 can suppress apoptosis, and therefore cells lacking IGF-1Rs or having compromised IGF-1R signaling pathways may give rise to tumor cells that selectively die via apoptosis (Long *et al.*, *Cancer Res.*, 55:1006-1009 (1995)). Furthermore, it has recently become evident that alterations in IGF signaling in the context of other disease states, such as diabetes, may be responsible for exacerbating the complications of retinopathy (Smith *et al.*, *Science*, 276:1706-1709 (1997)) and nephropathy (Horney *et al.*, *Am. J Physiol.* 274: F1045-F1053 (1998)).

IGF-1 *in vivo* is mostly found in complex with a family of at least six serum proteins known as IGFBPs (Jones and Clemmons, *supra*; Bach and Rechler, *Diabetes Reviews*, 3: 38-61 (1995)), that modulate access of the IGFs to the IGF-1R. They also regulate the concentrations of IGF-1 and IGF-2 in the circulation and at the level of the tissue IGF-1R (Clemmons *et al.*, *Anal. NY Acad. Sci. USA*, 692:10-21 (1993)). The IGFBPs bind IGF-1 and IGF-2 with varying affinities and specificities (Jones and Clemmons, *supra*; Bach and Rechler, *supra*). For example, IGFBP-3 binds IGF-1 and IGF-2 with a similar affinity, whereas IGFBP-2 and IGFBP-6 bind IGF-2 with a much higher affinity than they bind IGF-1 (Bach and Rechler, *supra*; Oh *et al.*, *Endocrinology*, 132, 1337-1344 (1993)). The major carrier protein is IGFBP-3. Nothing is currently known about the stoichiometry of binding in these complexes of IGF-1 and its IGFBPs, due to the heterogeneous size of the complexes caused by glycosylation.

IGF-1 naturally occurs in human body fluids, for example, blood and human cerebral spinal fluid. Although IGF-1 is produced in many tissues, most circulating IGF-1 is believed to be synthesized in the liver. The IGFBPs are believed to modulate the biological activity of IGF-1 (Jones and Clemmons, *supra*), with IGFBP-1 (Lee *et al.*, *Proc. Soc. Exp. Biol. & Med.*, 204: 4-29 (1993)) being implicated as the primary

binding protein involved in glucose metabolism (Baxter, "Physiological roles of IGF binding proteins", in: Spencer (Ed.), Modern Concepts of Insulin-like Growth Factors (Elsevier, New York, 1991), pp. 371-380). IGFBP-1 production by the liver is regulated by nutritional status, with insulin directly suppressing its production (Suikkari *et al.*, J. Clin. Endocrinol. Metab., **66**: 266-272 (1988)).

5       The function of IGFBP-1 *in vivo* is poorly understood. The administration of purified human IGFBP-1 to rats has been shown to cause an acute, but small, increase in blood glucose (Lewitt *et al.*, Endocrinology, **129**: 2254-2256 (1991)). The regulation of IGFBP-1 is somewhat better understood. It has been proposed (Lewitt and Baxter, Mol. Cell Endocrinology, **79**: 147-152 (1991)) that when blood glucose rises and insulin is secreted, IGFBP-1 is suppressed, allowing a slow increase in "free" IGF-1 levels that  
10       might assist insulin action on glucose transport. Such a scenario places the function of IGFBP-1 as a direct regulator of blood glucose.

15       In most cases, addition of exogenous IGFBP blunts the effects of IGF-I. For example, the growth-stimulating effect of estradiol on the MCF-7 human breast cancer cells is associated with decreased IGFBP-3 mRNA and protein accumulation, while the anti-estrogen ICI 182780 causes growth inhibition and increased IGFBP-3 mRNA and protein levels (Huynh *et al.*, J Biol. Chem., **271**:1016-1021 (1996); Oh *et al.*, Prog. Growth Factor Res., **6**:503-512 (1995)). It has also been reported that the *in vitro* inhibition of breast cancer cell proliferation by retinoic acid may involve altered IGFBP secretion by tumor cells or decreased circulating IGF-1 levels *in vivo* (LeRoith *et al.*, Ann. Int. Med., **122**:54-59 (1995); Oh *et al.*, (1995), *supra*). Contrary to this finding, treatment of MCF-7 cells with the anti-estrogen tamoxifen  
20       decreases IGF-IR signaling in a manner that is unrelated to decreased IGFBP production (Lee *et al.*, J Endocrinol., **152**:39 (1997)). Additional support for the general anti-proliferative effects of the IGFBPs is the striking finding that IGFBP-3 is a target gene of the tumor suppressor, p53 (Buckbinder *et al.*, Nature, **377**:646-649 (1995)). This suggests that the suppressor activity of p53 is, in part, mediated by IGFBP-3 production and the consequential blockade of IGF action (Buckbinder *et al.*, *supra*). These results indicate  
25       that the IGFBPs can block cell proliferation by modulating paracrine/autocrine processes regulated by IGF-1/IGF-2. A corollary to these observations is the finding that prostate-specific antigen (PSA) is an IGFBP-3-protease, which upon activation, increases the sensitivity of tumor cells to the actions of IGF-1/IGF-2 due to the proteolytic inactivation of IGFBP-3 (Cohen *et al.*, J. Endocr., **142**:407-415 (1994)). The IGFBPs complex with IGF-1/IGF-2 and interfere with the access of IGF-1/IGF-2 to IGF-IRs (Clemmons *et al.*, Anal. NY Acad. Sci. USA, **692**:10-21 (1993)). IGFBP-1, -2 and -3 inhibit cell growth following addition to cells  
30       *in vitro* (Lee *et al.*, J Endocrinol., **152**:39 (1997); Feyen *et al.*, J Biol. Chem., **266**:19469-19474 (1991)). Further, IGFBP-1 (McGuire *et al.*, J Natl. Cancer Inst., **84**:1335-1341(1992); Figueroa *et al.*, J Cell Physiol., **157**:229-236 (1993)), IGFBP-3 (Oh *et al.* (1995), *supra*; Pratt and Pollak, Biophys. Res. Commun., **198**:292-297 (1994)) and IGFBP-2 have all been shown to inhibit IGF-1 or estrogen-induced breast cancer  
35       cell proliferation at nanomolar concentrations *in vitro*. These findings support the idea that the IGFBPs are potent antagonists of IGF action. There is also evidence for a direct effect of IGFBP-3 on cells through its own cell surface receptor, independent of IGF interactions (Oh *et al.*, J Biol. Chem., **268**:14964-14971 (1993); Valentinis *et al.*, Mol. Endocrinol., **9**:361-367 (1995)). Taken together, these findings underscore the importance of IGF and IGF-IR as targets for therapeutic use.

IGFs have mitogenic and anti-apoptotic influences on normal and transformed prostate epithelial cells (Hsing *et al.*, Cancer Research, 56: 5146 (1996); Culig *et al.*, Cancer Research, 54: 5474 (1994); Cohen *et al.*, Hormone and Metabolic Research, 26: 81 (1994); Iwamura *et al.*, Prostate, 22: 243 (1993); Cohen *et al.*, J. Clin. Endocrin. & Metabol., 73: 401 (1991); Rajah *et al.*, J. Biol. Chem., 272: 12181 (1997)). Most circulating IGF-1 originates in the liver, but IGF bioactivity in tissues is related not only to levels of circulating IGFs and IGFBPs, but also to local production of IGFs, IGFBPs, and IGFBP proteases (Jones and Clemmons, *supra*). Person-to-person variability in levels of circulating IGF-1 and IGFBP-3 (the major circulating IGFBP (Jones and Clemmons, *supra*)) is considerable (Juul *et al.*, J. Clin. Endocrinol. & Metabol., 78: 744 (1994); Juul *et al.*, J. Clin. Endocrinol. & Metabol., 80: 2534 (1995)), and heterogeneity in serum IGF-1 levels appears to reflect heterogeneity in tissue IGF bioactivity. Markers relating to IGF-axis components can be used as a risk marker for prostate cancer, as PSA is likewise used (WO 99/38011).

Unlike most other growth factors, the IGFs are present in high concentrations in the circulation, but only a small fraction of the IGFs is not protein bound. For example, it is generally known that in humans or rodents, less than 1% of the IGFs in blood is in a "free" or unbound form (Juul *et al.*, Clin. Endocrinol., 44: 515-523 (1996); Hizuka *et al.*, Growth Regulation, 1: 51-55 (1991); Hasegawa *et al.*, J. Clin. Endocrinol. Metab., 80: 3284-3286 (1995)). The overwhelming majority of the IGFs in blood circulate as part of a non-covalently associated ternary complex composed of IGF-1 or IGF-2, IGFBP-3, and a large protein termed the acid-labile subunit (ALS). The ternary complex of an IGF, IGFBP-3, and ALS has a molecular weight of approximately 150,000 daltons, and it has been suggested that the function of this complex in the circulation may be to serve as a reservoir and buffer for IGF-1 and IGF-2, preventing rapid changes in free IGF-1 or IGF-2.

There has been much work identifying the regions on IGF-1 and IGF-2 that bind to the IGFBPs (Bayne *et al.*, J. Biol. Chem., 265: 15648-15652 (1990); Dubaquié and Lowman, Biochemistry, 38: 6386-6396 (1999); and U.S. Pat. Nos. 5,077,276; 5,164,370; and 5,470,828). For example, it has been discovered that the N-terminal region of IGF-1 and IGF-2 is critical for binding to the IGFBPs (U.S. Pat. Nos. 5,077,276; 5,164,370; and 5,470,828). Thus, the natural IGF-1 variant, designated des (1-3) IGF-1, binds poorly to IGFBPs.

A similar amount of research has been devoted to identifying the regions on IGF-1 and IGF-2 that bind to IGF-1R (Bayne *et al.*, *supra*; Oh *et al.*, Endocrinology (1993), *supra*). It was found that the tyrosine residues in IGF-1 at positions 24, 31, and 60 are crucial to the binding of IGF-1 to IGF-1R (Bayne *et al.*, *supra*). Mutant IGF-1 molecules where one or more of these tyrosine residues are substituted showed progressively reduced binding to IGF-1R. Bayne *et al.*, *supra*, also investigated whether such mutants of IGF-1 could bind to IGF-1R and to the IGFBPs. They found that quite different residues on IGF-1 and IGF-2 are used to bind to the IGFBPs from those used to bind to IGF-1R. It is therefore possible to produce IGF variants that show reduced binding to the IGFBPs, but, because they bind well to IGF-1R, show maintained activity in *in vitro* activity assays.

Also reported was an IGF variant that binds to IGFBPs but not to IGF receptors and therefore shows reduced activity in *in vitro* activity assays (Bar *et al.*, Endocrinology, 127: 3243-3245 (1990)). In this variant, designated (1-27, gly<sup>4</sup>, 38-70)-hIGF-1, residues 28-37 of the C-region of human IGF-1 are replaced by a four-residue glycine bridge.

Other truncated IGF-1 variants are disclosed. For example, in the patent literature, WO 96/33216 describes a truncated variant having residues 1-69 of authentic IGF-1. EP 742,228 discloses two-chain IGF-1 superagonists, which are derivatives of the naturally occurring, single-chain IGF-1 having an abbreviated C-region. The IGF-1 analogs are of the formula: BC<sup>n</sup>,A

5 wherein B is the B-region of IGF-1 or a functional analog thereof, C is the C-region of IGF-1 or a functional analog thereof, n is the number of amino acids in the C-region and is from about 6 to about 12, and A is the A-region of IGF-1 or a functional analog thereof.

10 Additionally, Cascieri *et al.*, Biochemistry, 27: 3229-3233 (1988) discloses four mutants of IGF-1, three of which have reduced affinity to IGF-1R. These mutants are: (Phe<sup>23</sup>,Phe<sup>24</sup>,Tyr<sup>25</sup>)IGF-1 (which is equipotent to human IGF-1 in its affinity to the Types 1 and 2 IGF and insulin receptors), (Leu<sup>24</sup>)IGF-1 and (Ser<sup>24</sup>)IGF-1 (which have a lower affinity than IGF-1 to the human placental IGF-1R, the placental insulin receptor, and the IGF-1R of rat and mouse cells), and desoctapeptide (Leu<sup>24</sup>)IGF-1 (in which the loss of aromaticity at position 24 is combined with the deletion of the carboxyl-terminal D-region of hIGF-1, which has lower affinity than (Leu<sup>24</sup>)IGF-1 for the IGF-1R and higher affinity for the insulin receptor). These four  
15 mutants have normal affinities for human serum binding proteins.

Bayne *et al.*, J. Biol. Chem., 263: 6233-6239 (1988) discloses four structural analogs of human IGF-1: a B-chain mutant in which the first 16 amino acids of IGF-1 were replaced with the first 17 amino acids of the B-chain of insulin, (Gln<sup>3</sup>,Ala<sup>4</sup>)IGF-1, (Tyr<sup>15</sup>,Leu<sup>16</sup>)IGF-1, and (Gln<sup>3</sup>,Ala<sup>4</sup>,Tyr<sup>15</sup>,Leu<sup>16</sup>)IGF-1. These studies identify some of the regions of IGF-1 that are responsible for maintaining high-affinity  
20 binding with the serum binding protein and the Type 2 IGF receptor.

In another study, Bayne *et al.*, J. Biol. Chem., 264: 11004-11008 (1988) discloses three structural analogs of IGF-1: (1-62)IGF-1, which lacks the carboxyl-terminal 8-amino-acid D-region of IGF-1; (1-27,Gly<sup>4</sup>,38-70)IGF-1, in which residues 28-37 of the C-region of IGF-1 are replaced by a four-residue glycine bridge; and (1-27,Gly<sup>4</sup>,38-62)IGF-1, with a C-region glycine replacement and a D-region deletion.  
25 Peterkofsky *et al.*, Endocrinology, 128: 1769-1779 (1991) discloses data using the Gly<sup>4</sup> mutant of Bayne *et al.*, *supra* (vol. 264).

Cascieri *et al.*, J. Biol. Chem., 264: 2199-2202 (1989) discloses three IGF-1 analogs in which specific residues in the A-region of IGF-1 are replaced with the corresponding residues in the A chain of insulin. The analogs are:

30 (Ile<sup>41</sup>,Glu<sup>45</sup>,Gln<sup>46</sup>,Thr<sup>49</sup>,Ser<sup>50</sup>,Ile<sup>51</sup>,Ser<sup>53</sup>,Tyr<sup>55</sup>,Gln<sup>56</sup>)IGF-1, an A-chain mutant in which residue 41 is changed from threonine to isoleucine and residues 42-56 of the A-region are replaced; (Thr<sup>49</sup>,Ser<sup>50</sup>,Ile<sup>51</sup>)IGF-1; and (Tyr<sup>55</sup>,Gln<sup>56</sup>)IGF-1.

Clemmons *et al.*, J. Biol. Chem., 265: 12210-12216 (1990) discloses use of IGF-1 analogs that have reduced binding affinity for either IGF-1R or binding proteins to study the ligand specificity of  
35 IGFBP-1 and the role of IGFBP-1 in modulating the biological activity of IGF-1.

WO 94/04569 discloses a specific binding molecule, other than a natural IGFBP, that is capable of binding to IGF-1 and can enhance the biological activity of IGF-1.

Peptides that bind to IGFBP-1, block IGF-1 binding to this binding protein, and thereby release "free-IGF" activity from mixtures of IGF-1 and IGFBP-1 have been recently described (Lowman *et al.*,  
40 Biochemistry, 37: 8870-8878 (1998); WO 98/45427 published October 15, 1998; Lowman *et al.*,

International Pediatric Nephrology Association, Fifth Symposium on Growth and Development in Children with Chronic Renal Failure (New York, March 13, 1999)). Also described is the natural molecule, des(1-3)IGF-1, which shows selectively reduced affinity for some of the IGF binding proteins, yet a maintained affinity for the IGF receptor (U.S. Pat. Nos. 5,077,276; 5,164,370; 5,470,828).

5        Exploitation of the interaction between IGF and IGFBP in screening, preventing, or treating disease has been limited, however, because of a lack of specific antagonists. To date, only one publication is known to exist that describes the application of an IGF-1/ IGF-2 antagonist as a potential therapeutic adjunct in the treatment of cancer (Pietrzkowski *et al.*, Cancer Res., 52: 6447-6451 (1992)). In that report, a peptide corresponding to the D-region of IGF- I was synthesized for use as an IGF-1/2 antagonist. This peptide  
10        exhibited questionable inhibitory activity against IGF-1. The basis for the observed inhibition is unclear, as the D-region does not play a significant role in IGF-1 R binding but rather, in IGF-1 binding to the insulin receptor (Cooke *et al.*, Biochem., 30:5484-5491 (1991); Bayne *et al.*, *supra* (Vol. 264); Yee *et al.*, Cell Growth and Different., 5:73-77 (1994)).

WO 00/23469 discloses the portions of IGFBP and IGF peptides that account for IGF-IGFBP  
15        binding, i.e., an isolated IGF binding domain of an IGFBP or modification thereof that binds IGF with at least about the same binding affinity as the full-length IGFBP. The patent publication also discloses an IGF antagonist that reduces binding of IGF to an IGF receptor, and/or binds to a binding domain of IGFBP.

Additionally, EP 639981 discloses pharmaceutical compositions comprising short peptides that  
20        function as IGF-1 receptor antagonists. The peptides used in the pharmaceutical compositions consist of less than 25 amino acids, comprise at least a portion of the C- or D-region from IGF-1, and inhibit IGF-1-induced autophosphorylation of IGF-1 receptors.

Polypeptides, including the IGF molecules, have a three-dimensional structure determined by the primary amino acid sequence and the environment surrounding the polypeptide. This three-dimensional structure establishes the activity, stability, binding affinity, binding specificity, and other biochemical  
25        attributes of the polypeptide. Thus, knowledge of the three-dimensional structure of a protein can provide much guidance in designing agents that mimic, inhibit, or improve its biological activity in soluble or membrane-bound forms.

The three-dimensional structure of a polypeptide may be determined in a number of ways. Many of the most precise methods employ x-ray crystallography (Van Holde, Physical Biochemistry (Prentice  
30        Hall: N.J., 1971), pp. 221-239). This technique relies on the ability of crystalline lattices to diffract x-ray or other forms of radiation. Diffraction experiments suitable for determining the three-dimensional structure of macromolecules typically require high-quality crystals. Unfortunately, such crystals have been unavailable for IGF-1 as well as many other proteins of interest. Crystals have been described for M-CSF (EP 668,914B1), CD40 ligand (WO 97/00895), and a BC2 Fab fragment (WO 99/01476), for example.

35        The crystallization of insulin is an intensively researched field, both with respect to work on structural analysis (Adams *et al.*, Nature, 224: 491 (1969)) and pharmaceutical applications. Examples of insulin crystal suspensions that are used therapeutically include suspensions of rhombohedral zinc-insulin crystals that are stable in the presence of 0.8 to 2.5% of zinc (based on the weight of insulin) at a neutral pH value and exhibit a delayed action, and isophane insulin protamine crystals, which are used in delayed  
40        action products in the form of small rods. A few other crystal modifications of insulin are furthermore

known, but these have hitherto been of interest only for X-ray structure analysis. Thus, zinc-free orthorhombic and monoclinic crystals have been obtained under acid pH conditions (Einstein and Low, Acta Crystallogr., 15: 32-34 (1962)). Smaller rhombic dodecahedra, which are to be classified in the cubic space group, have been obtained at the isoelectric point, also in the absence of zinc. Finally, a monoclinic crystal form of insulin has been obtained above the isoelectric point in the presence of zinc and in the presence of phenol or phenol derivatives. These crystals grow to a considerable size (up to 3 mm) within a few days and have sharp edges. Interestingly, these crystals have been found only on glass surfaces and not on the free surface of the solution. Crystal suspensions and other crystal forms of insulin preparations and insulin analogs are described, for example, in such representative patents as U.S. Pat. Nos. 4,959,351; 5,840,680; 5,834,422; 6,127,334; 5,952,297; 5,650,486; 5,898,028; 5,898,067; 5,948,751; 5,747,642; 5,597,893; 5,547,930; 5,534,488; 5,504,188; 5,461,031; and 5,028,587.

Various methods for preparing crystalline proteins and polypeptides are known in the art (McPherson *et al.*, "Preparation and Analysis of Protein Crystals," McPherson (Robert E. Krieger Publishing Company, Malabar, FL, 1989); Weber, *Advances in Protein Chemistry*, 41: 1-36 (1991); US Pat Nos. 4,672,108 and 4,833,233). Although there are multiple approaches to crystallizing polypeptides, no single set of conditions provides a reasonable expectation of success, especially when the crystals must be suitable for x-ray diffraction studies. Significant effort is required to obtain crystals of sufficient size and resolution to provide accurate information regarding the structure. For example, once a protein of sufficient purity is obtained, it must be crystallized to a size and clarity that is useful for x-ray diffraction and subsequent structure resolution. Further, although the amino acid sequence of a target protein may be known, this sequence information does not allow an accurate prediction of the crystal structure of the protein. Nor does the sequence information afford an understanding of the structural, conformational, and chemical interactions between a ligand such as an IGFBP and its protein target. Thus, although crystal structures can provide a wealth of valuable information in the field of drug design and discovery, crystals of certain biologically relevant compounds such as IGF-1 are not readily available to those skilled in the art. High-quality, diffracting crystals of IGF-1 would assist the determination of its three-dimensional structure.

Generation of specific IGF-1 antagonists has been restricted, at least in part, because of difficulties in studying the structure of IGF and IGFBPs. Due to the inability to obtain crystals of IGF-1 suitable for diffraction studies, for example, an extrapolation of IGF-1 structure based on the crystal structure of porcine insulin was the most important structural road map for IGF-1 available (Blundell *et al.*, Proc. Natl. Acad. Sci. USA, 75:180- 184 (1978)). See also Blundell *et al.*, Fed. Proc., 42: 2592-2597 (1983), which discloses tertiary structures, receptor binding, and antigenicity of IGFs. Based on studies of chemically modified and mutated IGF-1, a number of common residues between IGF- I and insulin have been identified as being part of the IGF-IR-insulin receptor contact site, in particular, the aromatic residues at positions 23-25.

Using NMR and restrained molecular dynamics, the solution structure of IGF-1 was recently reported (Cooke *et al.*, *supra*). The resulting minimized structure was shown to better fit the experimental findings on modified IGF-1, as well as the extrapolations made from the structure-activity studies of insulin. Further, De Wolf *et al.*, Protein Sci., 5: 2193-2202 (1996) discloses the solution structure of a mini-IGF-1. Sato *et al.*, Int. J. Pept. Protein Res., 41: 433-440 (1993) discloses the three-dimensional structure of IGF-1 determined by 1H-NMR and distance geometry. Laajoki *et al.*, J. Biol. Chem., 275: 10009-10015 (2000)



discloses the solution structure and backbone dynamics of long-[Arg(3)]IGF-1. See also Laajoki *et al.*, *FEBS Lett.*, **420**: 97-102 (1997)). The small number of NMR models available for IGF-1 are not very well defined, as there are large RMSDs between the backbone atoms of the helical segments. The best NMR model is of IGF-2 in which three alpha-helices are shown. See Torres *et al.*, *J. Mol. Biol.*, **248**: 385-401 (1995), which discloses the solution structure of human IGF-2 and its relationship to receptor and binding protein interactions. In all structures, the C- and D-regions are very poorly defined.

In addition to providing structural information, crystalline polypeptides provide other advantages. For example, the crystallization process itself further purifies the polypeptide and satisfies one of the classical criteria for homogeneity. In fact, crystallization frequently provides unparalleled purification quality, removing impurities that are not removed by other purification methods such as HPLC, dialysis, conventional column chromatography, etc. Moreover, crystalline polypeptides are often stable at ambient temperatures and free of protease contamination and other degradation associated with solution storage. Crystalline polypeptides may also be useful as pharmaceutical preparations. Finally, crystallization techniques in general are largely free of problems such as denaturation associated with other stabilization methods (e.g. lyophilization). Thus, there exists a significant need for preparing IGF-1 compositions in crystalline form and determining their three-dimensional structure. The present invention fulfills this and other needs. Once crystallization has been accomplished, crystallographic data provides useful structural information that may assist the design of peptides that may serve as agonists or antagonists. In addition, the crystal structure provides information useful to map the receptor-binding domain, which could then be mimicked by a small non-peptide molecule that may serve as an antagonist or agonist. Also, findings regarding the detergent's inhibition of the binding of IGFBP to IGF-1 can be used to identify new IGF-1 agonists.

#### Summary of the Invention

Accordingly, the invention is as claimed. IGF-1 has been crystallized and its structure determined using multiwavelength anomalous diffraction (MAD) at 1.8 angstroms resolution by exploiting the anomalous scattering of a single bromide ion and six of the seven sulfur atoms of IGF-1. The C-region of IGF-1, which is ordered in the crystal structure, forms a type II beta-turn and mediates a crystal packing interaction across a crystallographic dyad. The solution state of IGF-1 was characterized by analytical ultracentrifugation, and the results indicate that IGF-1 exists primarily as a monomer at neutral pH, with only a slight tendency to dimerize at millimolar concentrations. A molecule of detergent, N, N-bis(3-D-gluconamidopropyl)-deoxycholine (deoxy big CHAPS), mediates a crystal packing contact between symmetry-related molecules. Biophysical and biochemical data show that the N, N-bis(3-D-gluconamidopropyl)-deoxycholine binds to IGF-1 specifically and blocks binding of IGFBP-1 and IGFBP-3.

Accordingly, in one aspect, the invention provides a crystal formed by IGF-1 that diffracts x-ray radiation to produce a diffraction pattern representing the three-dimensional structure of the IGF-1. Preferably this crystal has approximately the following cell constants  $a=31.831$  Å,  $b=71.055$  Å,  $c=65.995$  Å, and a space group of C222<sub>1</sub>. Also preferably, the IGF-1 contains an A-, B-, C-, and D-region and forms a

dimer in the crystal, and further preferred is the crystal comprising a receptor binding site at the dimer interface.

The invention also provides a composition comprising the above crystal. Preferably in this composition the IGF-1 is biologically active when resolubilized. The invention further provides a method of treating a mammal suffering from an agonist disorder, preferably a human patient, said method comprising administering to said mammal an effective amount of the above resolubilized composition.

The invention also provides a method of crystallizing IGF-1 comprising the steps of:

(a) mixing an aqueous solution comprising IGF-1 with a reservoir solution comprising a precipitant to form a mixed volume; and

(b) crystallizing the mixed volume.

The invention also provides crystalline IGF-1 produced by the above method.

Additionally, the invention provides a method for determining a three-dimensional structure of IGF-1 comprising:

(a) crystallizing the IGF-1;

(b) irradiating the crystalline IGF-1 to obtain a diffraction pattern characteristic of the crystalline IGF-1; and

(c) transforming the diffraction pattern into the three-dimensional structure of the IGF-1.

Further, the invention provides a machine-readable data storage medium comprising a data storage material encoded with machine-readable data that, when read by an appropriate machine, displays a three-dimensional representation of a crystal of a molecule comprising IGF-1.

In further aspects, the invention provides an IGF-1 crystal with the structural coordinates shown in Appendix 1.

Additionally, the invention provides a method of using a three-dimensional structure of IGF-1 derived from an IGF-1 crystal wherein the three-dimensional structure of IGF-1 includes an IGF-1 receptor-binding region, the method comprising identifying compounds having structures that interact with the receptor-binding region of the three-dimensional structure of IGF-1 and function as an IGF-1 agonist or antagonist. Preferably in such method the three-dimensional structure of IGF-1 includes alpha-carbon coordinates substantially the same as those of the structural information presented in Appendix 1.

In another aspect, the invention provides a method of identifying IGF-1 agonists or antagonists comprising the steps of:

(a) crystallizing IGF-1 to form IGF-1 crystals, the IGF-1 crystals containing a group of amino acid residues defining an IGF-1 receptor-binding region;

(b) irradiating the IGF-1 crystals from step (a) to obtain a diffraction pattern of the IGF-1 crystals;

(c) determining a three-dimensional structure of IGF-1 from the diffraction pattern, the structure

including an IGF-1 receptor-binding region; and

(d) identifying an IGF-1 agonist or antagonist having a three-dimensional structure that functionally duplicates essential IGF receptor-binding, solvent-accessible residues presenting the three-dimensional structure of the IGF-1 receptor-binding region, said IGF-1 agonist or antagonist having altered signal transduction capacity to IGF-1-responsive cells, as compared to IGF-1.

Preferably, in this method the solvent-accessible residues do not participate in formation of the IGF-1 interface.

According to certain further aspects, the invention includes a method of designing a compound, such as a peptidomimetic, that mimics the 3-dimensional surface structure of IGF-1 comprising the steps of:

- (a) determining the 3-dimensional structure of the IGF-1; and
- (b) designing a compound that mimics the 3-dimensional surface structure of the IGF-1.

According to a further embodiment, the invention provides a method for identifying a peptidomimetic that binds IGF-1 and blocks binding of an IGFBP or a receptor that binds to IGF-1 comprising the steps of:

- (a) searching a molecular structure database with the structural parameters or structural coordinates provided in Appendix 1; and
- (b) selecting a molecule from the database that mimics the structural parameters or structural coordinates of the IGF-1.

The invention also provides a method for determining at least a portion of a three-dimensional structure of a molecular complex, said complex comprising IGF-1 and said method comprising the steps of:

- (a) determining the structural coordinates of a crystal of IGF-1;
- (b) calculating phases from the structural coordinates;
- (c) calculating an electron density map from the phases obtained in step (b); and
- (d) determining the structure of at least a portion of the complex based on said electron density map.

Preferably the structural coordinates used in step (a) are substantially the same as those described in Appendix 1 or describe substantially the same crystal as the coordinates in Appendix 1.

The invention also provides a method for evaluating the ability of a chemical entity to associate with IGF-1 or a complex thereof, the method comprising the steps of:

- (a) employing computational or experimental means to perform a fitting operation between the chemical entity and the IGF-1 or complex thereof, thereby obtaining data related to the association; and
- (b) analyzing the data obtained in step (a) to determine the characteristics of the association between the chemical entity and the IGF-1 or complex thereof.

The invention also provides a chemical entity identified by the above method that interferes with the *in vivo* or *in vitro* association between IGF-1 and its receptor or between IGF-1 and at least one of its binding proteins, or associates with a binding site on IGF-1.

Also provided is a heavy-atom derivative of a crystallized form of IGF-1.

The invention also comprises a method of computationally or experimentally evaluating a chemical entity to obtain information about its association with one or more binding sites of IGF-1 using a crystal of IGF-1 having the structural coordinates described in Appendix 1.

Any peptide analogs and other chemical entities identified using the above methods of the present invention are useful in the therapeutic methods described herein and as pharmaceutical compositions.

The invention also provides a method of identifying indirect agonists of IGF-1 comprising the steps of:

- (a) comparing the ability of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine to inhibit binding of IGFBP-1 or IGFBP-3 to IGF-1 with the ability of a candidate indirect agonist of IGF-1 to so inhibit binding; and
- (b) determining whether the candidate agonist inhibits such binding at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

In a preferred embodiment, the comparison is accomplished by competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and the candidate agonist. In a more preferred embodiment, inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholamine or the candidate agonist with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based ELISA assay.

The invention further provides a method of identifying indirect agonists of IGF-1 comprising co-crystallizing a candidate indirect agonist of IGF-1 with IGF-1 to form a co-crystalline structure and determining if the candidate agonist binds to one or both of two patches on IGF-1, wherein one patch has the amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and the second patch has the amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55, and wherein binding occurs if there is at least one contact between each listed amino acid residue of a given patch and the candidate agonist that is less than or equal to 6 angstroms in the co-crystalline structure. In preferred embodiments, the candidate agonist inhibits binding of IGFBP-1 or -3 to IGF-1 at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. More preferred is the method wherein inhibition of binding is measured using a competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and the candidate agonist. Most preferred is the method wherein inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholamine or the candidate agonist with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based ELISA assay.

Also provided herein is a method for treating an IGF-1 agonist disorder in a mammal comprising administering to the mammal an effective amount of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

Further provided herein is a co-crystalline complex of IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

#### Brief Description of the Drawings

Figure 1 aligns the sequences of IGF-1 (SEQ ID NO:1), IGF-2 (SEQ ID NO:2), and insulin (SEQ ID NO:3). The A-, B-, and C-chains of insulin (and the sequences of IGF-1 and IGF-2 corresponding thereto) are shown respectively in bold, underlined, and italicized text. The three aromatic residues are shown by outlining the text. The residues marked with a (!) have been demonstrated to be important for binding to the IGF-1 receptor. The residues marked with a "\*" have been shown to be important for binding to IGFBP-1 and IGFBP-3. The carboxyl-terminal residues comprising the D-region of IGF-1 and IGF-2 are depicted in regular type.

Figure 2 is a ribbon diagram of IGF-1 showing the backbone fold. In the Ramachandran plot, 97.7% is most favored and 2.3% is allowed.

Figure 3 is a ribbon diagram of both IGF-1 (left structure) and insulin (right structure).

Figure 4 is a ribbon diagram of IGF-1 showing that the detergent used in the reservoir solution, (N, N-bis(3-D-gluconamidopropyl)-deoxycholine), binds into a small hydrophilic cleft at the base of the B-helix. The detergent is represented by lighter gray structures than the IGF-1 structures.

Figure 5 is a ribbon diagram of IGF-1 as a dimer, with the detergent shown in lighter gray.

Figure 6 is a ribbon diagram of IGF-1 as a dimer, showing that the residues important for receptor binding (indicated by ring structures in the center portion of the figure) cluster at the dimer interface. The detergent is shown in lighter gray at the outer portions of the figure.

Figures 7A and 7B are ribbon diagrams of IGF-1 demonstrating, as does Fig. 4, that the detergent used in the reservoir solution (N, N-bis(3-D-gluconamidopropyl)-deoxycholine), shown in stick form, binds into a small hydrophilic cleft at the base of the B-helix. In Fig. 7A the detergent head group is inserted into the cleft lined by residues Leu 5, Phe 16, Val 17, Leu 54, and Leu 57. The various shades of gray are according to the alanine-scanning mutagenesis results of Dubaquié and Lowman, *supra*, with the Phe 16, Val 17, and Leu 5 regions indicating a 5-10 fold reduction, the Glu 3 region a 10-100 fold reduction, and the Pro 63 and Pro 63' regions a >100 fold reduction in affinity for IGFBP-1. The black part at the far right corresponds to the symmetry-related IGF-1 molecule that forms the crystallographic dimer. The circle near Leu 54 indicates the C10 atom of the detergent, which differs from another detergent (3-((3-cholamidopropyl) dimethylammonio)-1-propane sulfonate; or CHAPS) by having a hydroxyl group at this position. Fig. 7B shows the view from the opposite surface of the detergent and depicts the interactions of the detergent molecule with a symmetry-related IGF-1 molecule. As in Fig. 7A the various shades of gray are according to the alanine-scanning mutagenesis results of Dubaquié and Lowman, *supra*, with the group near Gln 15 indicating a 5-10 fold reduction, the far left medium gray molecules, the Leu 10 region molecules, and the far right medium gray region indicating a 10-100 fold reduction, and the black regions at Phe 49 and Gly 7 indicating a >100 fold reduction in affinity for IGFBP-1. The black regions to the right of the detergent molecule correspond to the symmetry-related IGF-1 molecule that forms the crystallographic dimer. The circle near Gln 15 indicates the C10 atom of the detergent, as noted above for Fig. 7A. This figure was prepared using the program INSIGHT (MSI, San Diego, CA).

Figure 8 shows a graph resulting from a detergent/IGFBP competition binding study. In this experiment, N, N-bis(3-D-gluconamidopropyl)-deoxycholine was used as a competitive inhibitor of IGF-1 binding to immobilized IGFBP-1 (open circles) or IGFBP-3 (open squares). As a positive control, soluble IGFBP-1 (solid circles) or IGFBP-3 (solid squares) was used as a competitive inhibitor of IGF-1 binding to immobilized IGFBP-1 or IGFBP-3, respectively. Each data point represents the average of three independent experiments.

Figure 9A shows a non-linear least-squares analysis of sedimentation equilibrium data for IGF-1 in solution. Data collected at rotor speeds of 30,000 rpm (open triangles) and 35,000 rpm (open squares) were fit as an ideal monomer-dimer self-association model. The solid lines are the fits of the data. Figure 9B shows the residuals plotted for both rotor speeds after accounting for the data by the fitting procedure. They are randomly distributed around zero, indicating that the monomer-dimer model is correct for this interaction.

Figure 10A shows a ribbon diagram determined by NMR of a complex of IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine), and Figure 10B shows a ribbon diagram determined by NMR of a complex of IGF-1 bound to a phage-derived IGF-1 antagonist peptide designated IGF-F1-1.

## Description of the Preferred Embodiments

### A. Definitions

As used herein, "IGF-1" refers to human insulin-like growth factor-1 unless otherwise noted, and has the human native mature IGF-1 sequence without a N-terminal methionine, as described, for example, by EP 230,869 published August 5, 1987; EP 128,733 published December 19, 1984; or EP 288,451 published October 26, 1988.

An "IGFBP" or an "IGF binding protein" refers to a protein or polypeptide normally associated with or bound or complexed to IGF-1, whether or not it is circulatory (*i.e.*, in serum or tissue). This definition includes IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, Mac 25 (IGFBP-7), and prostacyclin-stimulating factor (PSF) or endothelial cell-specific molecule (ESM-1), as well as other proteins with high homology to IGFBPs. Mac 25 is described, for example, in Swisshelm *et al.*, Proc. Natl. Acad. Sci. USA, 92: 4472-4476 (1995) and Oh *et al.*, J. Biol. Chem., 271: 30322-30325 (1996). PSF is described in Yamauchi *et al.*, Biochemical Journal, 303: 591-598 (1994). ESM-1 is described in Lassalle *et al.*, J. Biol. Chem., 271: 20458-20464 (1996). For other identified IGFBPs, see, *e.g.*, EP 375,438 published 27 June 1990; EP 369,943 published 23 May 1990; U.S. Pat. No. 5,258,287; WO 89/09268 published 5 October 1989; Wood *et al.*, Molecular Endocrinology, 2: 1176-1185 (1988); Brinkman *et al.*, The EMBO J., 7: 2417-2423 (1988); Lee *et al.*, Mol. Endocrinol., 2: 404-411 (1988); Brewer *et al.*, BBRC, 152: 1289-1297 (1988); EP 294,021 published 7 December 1988; Baxter *et al.*, BBRC, 147: 408-415 (1987); Leung *et al.*, Nature, 330: 537-543 (1987); Martin *et al.*, J. Biol. Chem., 261: 8754-8760 (1986); Baxter *et al.*, Comp. Biochem. Physiol., 91B: 229-235 (1988); WO 89/08667 published 21 September 1989; WO 89/09792 published 19 October 1989; and Binkert *et al.*, EMBO J., 8: 2497-2502 (1989). IGFBP-1 and IGFBP-3 bind to different residues of IGF-1.

As used herein, "human IGF-1 receptor" or just "IGF-1 receptor" refers to any receptor for IGF-1 found in humans and includes the Type 1 and Type 2 IGF receptors in humans to which human IGF-1 binds, such as the placental IGF-1R, *etc.*

An "indirect agonist of IGF-1" is a molecule that releases IGF-1 *in situ* from IGFBP-3 or IGFBP-1 so that the IGF-1 released is active and interacts with its receptor.

"Peptides" are molecules having at least two amino acids and include polypeptides having at least about 60 amino acids. Preferably, the peptides have about 10 to about 60 amino acids, more preferably about 10-25, and most preferably about 12-25 amino acids. The definition includes linear and cyclic peptides, peptide derivatives, their salts, or optical isomers.

As used herein, "mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic, and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, sheep, pigs, cows, *etc.* The preferred mammal herein is a human. The term "non-adult" refers to mammals that are from perinatal age (such as low-birth-weight infants) up to the age of puberty, the latter being those that have not yet reached full growth potential.

As used herein, the term "treating" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to having the disorder or diagnosed with the disorder or those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with an IGF-1 agonist ("agonist disorder") or antagonist ("antagonist disorder"). This includes chronic and acute disorders or diseases including those pathological conditions that predispose the mammal to the disorder in question. The disorder being treated may be a combination of two or more of the agonist or antagonist disorders listed below.

Non-limiting examples of antagonist disorders include benign and malignant tumors, leukemias and lymphoid malignancies, neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders, and inflammatory, angiogenic and immunologic disorders, diabetic complications such as diabetic retinopathies or neuropathies, age-related macular degeneration, ophthalmic surgery such as cataract extraction, a corneal transplant, glaucoma filtration surgery and keratoplasty, surgery to correct refraction, i.e., a radial keratotomy, also in sclera macular holes and degeneration, retinal tears, vitreoretinopathy, miscellaneous disorders, cataract disorders of the cornea such as the sequelae of radial keratotomy, dry eye, viral conjunctivitis, ulcerative conjunctivitis, wounds such as corneal epithelial wounds, Sjogren's syndrome, retinal disorders such as macular and retinal edema, vision-limited scarring, retinal ischemia, and proliferative vitreous retinopathy.

More preferably, such antagonist disorders include diabetic complications exacerbated by IGF-1, ischemic injury, and diseases associated with undesirable cell proliferation such as cancer, restenosis, and asthma. If the disorder is a diabetic complication exacerbated by IGF-1, such complication can include diabetic retinopathy or diabetic nephropathy. The efficacy of the treatment can be evidenced by a reduction in clinical manifestations or symptoms, including, for example, improved renal clearance, improved vision, or a reduction in the amount of IGF-1 available for binding to an IGF-1 receptor. If the disorder is an ischemic injury, it can include strokes, myocardial ischemia, and ischemic injury to the kidneys.

Examples of agonist disorders for purposes herein include any condition that would benefit from treatment with an IGF-1, including but not limited to, for example, lung diseases, hyperglycemic disorders as set forth below, renal disorders, such as acute and chronic renal insufficiency, end-stage chronic renal failure, glomerulonephritis, interstitial nephritis, pyelonephritis, glomerulosclerosis, *e.g.*, Kimmelstiel-Wilson in diabetic patients and kidney failure after kidney transplantation, obesity, GH-insufficiency, Turner's syndrome, Laron's syndrome, short stature, undesirable symptoms associated with aging such as obesity and increased fat mass-to-lean ratios, immunological disorders such as immunodeficiencies including decreased CD4 counts and decreased immune tolerance or chemotherapy-induced tissue damage, bone marrow transplantation, diseases or insufficiencies of cardiac structure or function such as heart dysfunctions and congestive heart failure, neuronal, neurological, or neuromuscular disorders, *e.g.*, peripheral neuropathy, multiple sclerosis, muscular dystrophy, or myotonic dystrophy, and catabolic states associated with wasting caused by any condition, including, *e.g.*, trauma or wounding, or infection such as with a bacterium or human virus such as HIV, wounds, skin disorders, gut structure and function that need restoration, and so forth. The preferred agonist disorders targeted for treatment herein are diabetes and

obesity, heart dysfunctions, AIDS-related wasting, kidney disorders, neurological disorders, whole body growth disorders, and immunological disorders.

As used herein, the term "hyperglycemic disorders" refers to all forms of diabetes and disorders resulting from insulin resistance, such as Type I and Type II diabetes, as well as severe insulin resistance, hyperinsulinemia, and hyperlipidemia, *e.g.*, obese subjects, and insulin-resistant diabetes, such as Mendenhall's Syndrome, Werner Syndrome, leprechaunism, lipotrophic diabetes, and other lipotrophies. The preferred hyperglycemic disorder is diabetes, especially Type I and Type II diabetes. "Diabetes" itself refers to a progressive disease of carbohydrate metabolism involving inadequate production or utilization of insulin and is characterized by hyperglycemia and glycosuria.

"Biologically active" IGF-1 refers to IGF-1 that exhibits a biological property conventionally associated with an IGF-1 agonist or antagonist, such as a property that would allow treatment of one or more of the disorders listed above.

The term "effective amount" refers to an amount of IGF-1 or a peptidomimetic or other compound, including chemical entities, effective to treat a disease or disorder in a mammal. In the case of cancer, for example, the effective amount of the peptide may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow at least to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; promote apoptosis; and/or relieve to some extent one or more of the symptoms associated with the disorder.

A "precipitant" is an agent in a reservoir solution that precipitates IGF-1 when mixed with an aqueous solution of IGF-1 and allowed to equilibrate so as to form IGF-1 crystals. Examples include chaotropic agents such as ammonium sulfate, polyethylene glycols (of a wide variety of molecular weights ranging, for example, from about 2000 to 20,000), sodium citrate, sodium cacodylate, or a mixture thereof.

A "reservoir solution" is a solution of a precipitant and any other ingredient needed to provide IGF-1 crystals, for example, a detergent such as C<sub>12</sub>E<sub>9</sub> (nonaethylene glycol monododecyl ether, nonaethylene glycol monolauryl ether, polyoxyethylene (9) ether), C<sub>12</sub>E<sub>8</sub> (octaethylene glycol monododecyl ether, octaethylene glycol monolauryl ether, polyoxyethylene (8) lauryl ether), dodecyl-beta-D-maltopyranoside, lauric acid sucrose ester, cyclohexyl-pentyl-beta-D-maltoside, nonaethylene glycol octylphenol ether, cetyltrimethylammonium bromide, decyl-beta-D-maltopyranoside, lauryldimethylamine oxide, cyclohexyl-pentyl-beta-D-maltoside, n-dodecylsulfo betaine, 3-(dodecyltrimethylammonio)propane-1-sulfonate, nonyl-beta-D-glucopyranoside, octyl-beta-D-thioglucopyranoside, OSG, N, N-dimethyldodecylamine-beta-oxide, methyl-6-O-(N-heptylcarbamoyl)-alpha-D-glycopyranoside, sucrose monocaproylate, heptyl-beta-D-thioglucopyranoside, octyl-beta-D-glucopyranoside, cyclohexyl-propyl-beta-D-maltoside, cyclohexylbutanoyl-N-hydroxyethylglucamide, n-decylsulfo betaine, 3-(decyldimethylammonio)propane-1-sulfonate, octanoyl-N-methylglucamide, hexyl-beta-D-glucopyranoside, and N, N-bis(3-D-gluconamidopropyl)-deoxycholine. Preferably, the detergent is N, N-bis(3-D-gluconamidopropyl)-deoxycholine.

"Recrystallization" refers to the procedure, after the initial crystals are grown and determined not to be very large or useful, of adding a substance to the crystals, such as methyl pentanediol, which has the effect of dissolving the crystals, but not diluting anything else much in the crystallization mixture. Then



over the course of several days, as the crystallization droplet re-equilibrates with its reservoir solution, the crystals regrow, but this time much larger and more well ordered.

The term "associating with" refers to a condition of proximity between IGF-1 and a chemical entity, or portions thereof. The association may be non-covalent, wherein the juxtaposition is energetically favored by hydrogen bonding, van der Waals interaction, or electrostatic interaction, or it may be a covalent association.

The term "binding site" refers to any or all of the sites where a chemical entity binds or associates with IGF-1.

The term "structural coordinates" refers to the coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of x-rays by the atoms (scattering centers) of a molecule in crystal form. The diffraction data can be used to calculate an electron density map of the repeating units of the crystal. Those skilled in the art will understand that the data obtained are dependent upon the particular system used, and hence, different coordinates may in fact describe the same crystal if such coordinates define substantially the same relationship as those described herein. An electron density map may be used to establish the positions of the individual atoms within the unit cell of the crystal.

Those of skill in the art understand that a set of structural coordinates determined by x-ray crystallography is not without standard error. Appendix 1 shows the atomic coordinates of IGF-1. For the purpose of this invention, any set of structural coordinates of IGF-1 that have a root mean square deviation of equivalent protein backbone atoms of less than about 2 Å when superimposed--using backbone atoms--on the structural coordinates in Appendix 1 shall be considered identical. Preferably, the deviation is less than about 1 Å and more preferably less than about 0.5 Å.

The term "heavy-atom derivatization" refers to a method of producing a chemically modified form of a crystallized IGF-1. In practice, a crystal is soaked in a solution containing heavy metal atom salts, or organometallic compounds, e.g., lead chloride, gold thiomalate, thimerosal, or uranyl acetate, which can diffuse through the crystal and bind to the surface of the protein. The location of the bound heavy metal atom(s) can be determined by x-ray diffraction analysis of the soaked crystal. This information can be used to generate the phase information used to construct the three-dimensional structure of the molecule.

The term "unit cell" refers to a basic shaped block. The entire volume of a crystal may be constructed by regular assembly of such blocks. Each unit cell comprises a complete representation of the unit of pattern, the repetition of which builds up the crystal.

The term "space group" refers to the arrangement of symmetry elements of a crystal.

The term "molecular replacement" refers to a method that involves generating a preliminary structural model of a crystal whose structural coordinates are unknown, by orienting and positioning a molecule whose structural coordinates are known, e.g., the IGF-1 coordinates in Appendix 1, within the unit cell of the unknown crystal, so as to best account for the observed diffraction pattern of the unknown crystal. Phases can then be calculated from this model, and combined with the observed amplitudes to give an approximated Fourier synthesis of the structure whose coordinates are unknown. This in turn can be subject to any of the several forms of refinement to provide a final accurate structure of the unknown crystal. (See, e.g., Lattman, E., "Use of the Rotation and Translation Functions," Methods in Enzymology, 115: 55-77 (1985); Rossman, ed., "The Molecular Replacement Method," Int. Sci. Rev. Ser. No. 13

(Gordon and Breach: New York, 1972)). Using the structural coordinates of IGF-1 provided by this invention, molecular replacement may be used to determine the structural coordinates of a crystalline co-complex, unknown ligand, mutant, or homolog, or of a different crystalline form of IGF-1. Additionally, the claimed crystal and its coordinates may be used to determine the structural coordinates of a chemical entity that associates with IGF-1.

The term "chemical entity" or "compound" as used herein means any molecule, molecular complex, compound, peptidomimetic, or fragment thereof that is not IGF-1. Preferably it is a molecule with high oral bioavailability, such as an organic chemical molecule, or a peptide.

#### B. Modes for Carrying out the Invention

The following detailed description of the invention encompasses the crystal structure of IGF-1, methods of making an IGF-1 crystal, and methods of using an IGF-1 crystal and its structural coordinates.

##### a. Crystal structure of IGF-1

The claimed invention provides crystals of IGF-1 as well as the structure of IGF-1 determined therefrom. Specifically, the claimed invention provides crystals of IGF-1 having approximately the following dimensions:  $a=31.831 \text{ \AA}$ ,  $b=71.055 \text{ \AA}$ ,  $c=65.995 \text{ \AA}$ ,  $\alpha=\beta=\gamma=90.000^\circ$ . It has a symmetry, or space group, of  $C222_1$ . The ribbon structure thereof is shown in Figure 2 having three helices, with the N-terminal B-region corresponding to residues 3-28, the C-region from residues 29-34, a stretch of poorly ordered residues from residues 35-40, and the A-region from residues 42-62. The D-region (residues 63-70) is essentially disordered. Figures 4 and 7 show that the detergent used in the crystallization binds into a small hydrophobic cleft at the base of the B-helix of the structure. The IGF-1 can form a dimer in the crystal, as shown in Fig. 5, wherein the two tails are positioned at the dimer interface. The buried surface area is  $689 \text{ \AA}^2/\text{monomer}$ , which is  $1378 \text{ \AA}^2$  total. The residues important for IGF-1R binding cluster at the dimer interface, as shown in Figure 6.

The characteristics of the claimed IGF-1 crystal are further described in the Example herein and the structural coordinates thereof are provided in Appendix 1.

##### b. Methods of making an IGF-1 crystal

In various embodiments, the claimed invention relates to methods of preparing crystalline forms of IGF-1 by first providing an aqueous solution comprising IGF-1. A reservoir solution comprising a precipitant is then mixed with a volume of the IGF-1 solution and the resultant mixed volume is then crystallized. In a preferred step the crystals are again dissolved and recrystallized. An example of a reagent that can be used for recrystallization is methyl pentanediol, which is preferred. The crystals are typically dissolved with this reagent in a small amount to minimize dilution effects of the other reagents and left to regrow for a period of time. In an optional step, the crystalline IGF-1 is isolated from the mixed volume. Preferably, the IGF-1 is obtained from a prokaryotic cell, more preferably a bacterial cell, most preferably *E. coli*. Preferably it is secreted into the periplasm and prepared as described in U.S. Pat. No. 5,723,310.

The concentration of IGF-1 in the aqueous solution may vary, but is preferably about 1 to 50 mg/ml, more preferably about 5 to 15 mg/ml. Similarly, precipitants used in the invention may vary, and may be selected from any precipitant known in the art. Preferably, the precipitant is selected from the group consisting of sodium citrate, ammonium sulfate, polyethylene glycol, sodium cacodylate, or a mixture thereof. More preferably the precipitant is polyethylene glycol buffered with sodium citrate or sodium

cacodylate. Any concentration of precipitant may be used in the reservoir solution; however, it is preferred that the concentration be about 20 to 25% if polyethylene glycol, and about 1 to 10 M if sodium citrate, ammonium sulfate, or sodium cacodylate. Preferably, the reservoir solution further comprises a detergent. Preferably, the detergent is present in an amount of about 10 to 50 mM. Also preferably the detergent is N, N-bis(3-D-gluconamidopropyl)-deoxycholine. The pH of the reservoir solution may also be varied, preferably between about 4 to 10, most preferably about 6.5.

One skilled in the art will understand that each of these parameters can be varied without undue experimentation and acceptable crystals will still be obtained. In practice, once the appropriate precipitating agents, buffers, or other experimental variables are determined for any given growth method, any of these methods or any other methods can be used to grow the claimed crystals. One skilled in the art can determine the variables depending upon one's particular needs.

Various methods of crystallization can be used in the claimed invention, including vapor diffusion, batch, liquid-bridge, or dialysis crystallization. Vapor diffusion crystallization is preferred. See, e.g. McPherson *et al.*, Preparation and Analysis of Protein Crystals, Glick, ed. (John Wiley & Co., 1982), pp. 82-159; Jancarik *et al.*, J. Appl. Crystallogr., 24: 409-411 (1991).

In vapor diffusion crystallization, a small volume (i.e., a few milliliters) of protein solution is mixed with a solution containing a precipitant. This mixed volume is suspended over a well containing a small amount, i.e. about 1 ml, of precipitant. Vapor diffusion from the drop to the well will result in crystal formation in the drop.

The dialysis method of crystallization utilizes a semipermeable size-exclusion membrane that retains the protein but allows small molecules (i.e. buffers and precipitants) to diffuse in and out. In dialysis, rather than concentrating the protein and the precipitant by evaporation, the precipitant is allowed to slowly diffuse through the membrane and reduce the solubility of the protein while keeping the protein concentration fixed.

The batch methods generally involve the slow addition of a precipitant to an aqueous solution of protein until the solution just becomes turbid; at this point the container can be sealed and left undisturbed for a period of time until crystallization occurs.

Thus, applicants intend that the claimed invention encompass any and all methods of crystallization. One skilled in the art can choose any of such methods and vary the parameters such that the chosen method results in the desired crystals.

The most preferred method of crystallization involves the method wherein the IGF-1, after isolation from the cell and formulation in, for example, an acetate, citrate, or succinate buffer, as described, for example, in U.S. Pat. No. 5,681,814 and WO 99/51272, is optionally desalted if necessary to a pH of about 4-5, preferably about 4.5, to form an aqueous solution. Then, a droplet of the aqueous solution is mixed with about 24% polyethylene glycol buffered to about pH 6.5 with either about 0.1M sodium citrate or about 0.1M sodium cacodylate and with about 1  $\mu$ l of about 1.4 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholine as detergent. This solution is then equilibrated by vapor diffusion crystallization with about 1 mL of about 24% polyethylene glycol buffered to about pH 6.5 with either about 0.1M sodium citrate or about 0.1M sodium cacodylate until crystallization droplets are formed, usually about 4-5 days.

Then about 2  $\mu$ l of about 100% methyl pentanediol is added to the crystallization droplets so as to dissolve the crystals overnight and thereby form new crystals, usually within a week's time.

The crystal structure was determined by combined anomalous scattering from intrinsic sulfur and fortuitous bromide ion as discussed in detail in the Example below.

5        c. Methods of using an IGF-1 crystal and its coordinates

The crystalline IGF-1 herein can be used for various purposes. For example, the crystallization process itself further purifies the IGF-1 to homogeneity. Thus, one such purpose is to provide a highly purified IGF-1 that can be used as a standard or control in a diagnostic setting, for example, as a molecular weight marker, or as an ELISA, radioassay, or radioreceptor assay control. Moreover, crystalline IGF-1 is  
10       stable at room temperature, can be lyophilized readily, and is less apt to degrade than less pure compositions.

In another use for the invention herein, crystals of IGF-1 of a size and quality to allow performance of x-ray diffraction studies enable those of skill in the art to conduct studies relating to the binding properties of IGF-1, as well as the binding properties of IGFBPs, IGF-1 receptors, and ALS that associate  
15       with the IGF-1.

Furthermore, structural information derived from a peptide crystal structure can be used for the identification of chemical entities, for example, small organic and bioorganic molecules such as peptidomimetics and synthetic organic molecules that bind IGF-1 and preferably block or prevent an IGF-1-mediated or -associated process or event, or that act as IGF-1 agonists. An exemplary approach to such a structure-based compound design is described in Structure Based Drug Design, Pandi Veerapandian, ed.  
20       (Marcell Dekker: New York 1997).

By way of example, having determined the three-dimensional structure of the IGF-1, the skilled artisan constructs a model of the IGF-1 such as those depicted in Figures 2 and 5. Since every atom of a peptide or polypeptide can be depicted as a sphere of the appropriate van der Waals radius, a detailed  
25       surface map of the folded IGF-1 can be constructed. The surface that results is known as the van der Waals surface. The "solvent-accessible surface" is the surface that is accessible to a chemical probe, a water molecule herein, and is constructed by rolling a water molecule of appropriate radius on the outside of the peptide maintaining contact with the van der Waals surface. Those parts of the van der Waals surface that contact the surface of the water molecule define a continuous surface known as the "solvent-accessible  
30       surface." (Creighton, Thomas E., Proteins: structure and molecular properties, 2nd. ed. (W. H. Freeman and Company, 1984), pp227-229).

Such chemical entities presenting a solvent-accessible surface that mimics the solvent-accessible surface of the IGF-1 can be constructed by those skilled in the art. By way of example, the skilled artisan can search three-dimensional structural databases of compounds to identify those compounds that position  
35       appropriate functional groups in similar 3-dimensional structural arrangement, then build combinatorial chemistry libraries around such chemical entities to identify those with high affinity.

One approach enabled by this invention is the use of the structural coordinates of IGF-1 to design chemical entities that bind to or associate with IGF-1 and alter the physical properties of the chemical entities in different ways. Thus, properties such as, for example, solubility, affinity, specificity, potency,  
40       on/off rates, or other binding characteristics may all be altered and/or maximized.

One may design desired chemical entities by probing an IGF-1 crystal with a library of different entities to determine optimal sites for interaction between candidate chemical entities and IGF-1. For example, high-resolution x-ray diffraction data collected from crystals saturated with solvent allows the determination of where each type of solvent molecule adheres. Small molecules that bind tightly to those sites can then be designed and synthesized and tested for the desired activity. Once the desired activity is obtained, the molecules can be further altered to maximize desirable properties.

The invention also contemplates computational screening of small-molecule databases or designing of chemical entities that can bind in whole or in part to IGF-1. They may also be used to solve the crystal structure of mutants, co-complexes, or the crystalline form of any other molecule homologous to, or capable of associating with, at least a portion of IGF-1.

One method that may be employed for this purpose is molecular replacement. An unknown crystal structure, which may be any unknown structure, such as, for example, another crystal form of IGF-1, an IGF-1 mutant or peptide, or a co-complex with IGF-1, or any other unknown crystal of a chemical entity that associates with IGF-1 that is of interest, may be determined using the structural coordinates as set forth in Appendix 1. Co-complexes with IGF-1 may include, but are not limited to, IGF-1-IGFBP-3, IGF-1-IGFBP-3-ALS, IGF-1-receptor, IGF-1-peptide, or IGF-1-small molecule. This method will provide an accurate structural form for the unknown crystal far more quickly and efficiently than attempting to determine such information without the invention herein.

The information obtained can thus be used to obtain maximally effective inhibitors or agonists of IGF-1. The design of chemical entities that inhibit or agonize IGF-1 generally involves consideration of at least two factors. First, the chemical entity must be capable of physically or structurally associating with IGF-1. The association may be any physical, structural, or chemical association, such as, for example, covalent or noncovalent bonding, or van der Waals, hydrophobic, or electrostatic interactions.

Second, the chemical entity must be able to assume a conformation that allows it to associate with IGF-1. Although not all portions of the chemical entity will necessarily participate in the association with IGF-1, those non-participating portions may still influence the overall conformation of the molecule. This in turn may have a significant impact on the desirability of the chemical entity. Such conformational requirements include the overall three-dimensional structure and orientation of the chemical entity in relation to all or a portion of the binding site.

The potential inhibitory or binding effect of a chemical entity on IGF-1 may be analyzed prior to its actual synthesis and testing by the use of computer-modeling techniques. If the theoretical structure of the given chemical entity suggests insufficient interaction and association between it and IGF-1, the need for synthesis and testing of the chemical entity is obviated. However, if computer modeling indicates a strong interaction, the molecule may then be synthesized and tested for its ability to bind to IGF-1. Thus, expensive and time-consuming synthesis of inoperative compounds may be avoided.

An inhibitory or other binding compound of IGF-1 may be computationally evaluated and designed by means of a series of steps in which chemical entities or fragments are screened and selected for their ability to associate with the individual binding sites of IGF-1.

Thus, one skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with IGF-1. This process may begin by visual inspection of, for example, the

binding site on a computer screen based on the IGF-1 coordinates in Appendix 1. Selected fragments or chemical entities may then be positioned in a variety of orientations, or "docked," within an individual binding pocket of IGF-1. Docking may be accomplished using software such as Quanta and Sybyl, followed by energy minimization and molecular dynamics with standard molecular mechanics force fields, such as

5 CHARMM and AMBER.

Specialized computer programs may be of use for selecting interesting fragments or chemical entities. These programs include, for example, GRID, available from Oxford University, Oxford, UK; MCSS or CATALYST, available from Molecular Simulations, Burlington, MA; AUTODOCK, available from Scripps Research Institute, La Jolla, CA; DOCK, available from University of California, San

10 Francisco, CA, and XSITE, available from University College of London, UK.

Once suitable chemical entities or fragments have been selected, they can be assembled into an inhibitor or agonist. Assembly may be by visual inspection of the relationship of the fragments to each other on the three-dimensional image displayed on a computer screen, in relation to the structural coordinates disclosed herein.

Alternatively, one may design the desired chemical entities *de novo*, experimentally, using either an empty binding site or optionally including a portion of a molecule with desired activity. Thus, for example, one may use solid-phase screening techniques where either IGF-1 or a fragment thereof, or candidate chemical entities to be evaluated, are attached to a solid phase, thereby identifying potential binders for further study.

15

Basically, any molecular modeling techniques may be employed in accordance with the invention; these techniques are known, or readily available to those skilled in the art. It will be understood that the methods and compositions disclosed herein can be used to identify, design, or characterize not only entities that will associate or bind to IGF-1, but alternatively to identify, design, or characterize entities that, like IGF-1, will bind to the receptor, thereby disrupting the IGF-1-receptor interaction. The claimed invention is

20 intended to encompass these methods and compositions broadly.

Once a compound has been designed or selected by the above methods, the efficiency with which that compound may bind to IGF-1 may be tested and modified for the maximum desired characteristic(s) using computational or experimental evaluation. Various parameters can be maximized depending on the desired result. These include, but are not limited to, specificity, affinity, on/off rates, hydrophobicity,

25 solubility, and other characteristics readily identifiable by the skilled artisan.

Additionally, the invention is useful for the production of small-molecule drug candidates. Thus, the claimed crystal structures may be also used to obtain information about the crystal structures of complexes of the IGF-1 and small-molecule inhibitors. For example, if the small-molecule inhibitor is co-crystallized with IGF-1, then the crystal structure of the complex can be solved by molecular replacement

30 using the known coordinates of IGF-1 for the calculation of phases. Such information is useful, for example, for determining the nature of the interaction between the IGF-1 and the small-molecule inhibitor, and thus may suggest modifications that would improve binding characteristics such as affinity, specificity, and kinetics.

35

d. Other methods

The invention herein is also useful in providing a method of identifying indirect agonists of IGF-1 based on the inhibitory properties of N, N-bis(3-D-gluconamidopropyl)-deoxycholine with respect to IGFBPs. This method comprises the steps of: comparing the ability of N, N-bis(3-D-gluconamidopropyl)-deoxycholine to inhibit binding of IGFBP-1 or -3 to IGF-1 with the ability of a candidate IGF-1 indirect agonist to inhibit such binding; and determining whether the candidate IGF-1 indirect agonist can inhibit such binding at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholine can so inhibit the binding.

Preferably the comparison is accomplished by competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholine and the candidate IGF-1 indirect agonist, using IC<sub>50</sub> to measure ability to inhibit IGFBP binding. In a more preferred embodiment, inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholine or the candidate agonist molecule with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based assay, such as an ELISA.

The invention further provides a method of identifying indirect agonists of IGF-1 comprising co-crystallizing the candidate agonist with IGF-1 to form a co-crystalline structure and determining if the candidate agonist molecule binds to one or both of two patches on IGF-1. The first patch contains the amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and the second patch contains the amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55. For purposes herein, binding means that there is at least one contact between each listed amino acid residue of a given patch and the candidate agonist molecule that is less than or equal to 6 angstroms in the co-crystalline structure. Such a candidate agonist molecule will have the property of inhibiting binding of IGFBP-1 or IGFBP-3 to IGF-1. The preferred such candidate agonist molecule will inhibit binding of IGFBP-1 or -3 to IGF-1 at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholine. More preferred is the method wherein inhibition of binding is measured using a competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholine and the candidate agonist molecule. Most preferred is the method wherein inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholine or the candidate agonist molecule with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based ELISA assay.

The N, N-bis(3-D-gluconamidopropyl)-deoxycholine detergent herein can be used as a template to perform design of small-molecule drugs that elicit the same effect as the detergent (compete with IGF-1 for IGFBP binding and subsequent disruption of the interaction of IGFBP with IGF-1 to free IGF-1 *in situ* so that it is active and will interact with the receptor. As opposed to the other detergents tested in the Examples below, N, N-bis(3-D-gluconamidopropyl)-deoxycholine lacks an oxygen atom at position C10. This region of the detergent is in close contact with the side-chain atoms of residues Leu 5, Leu 54, and Leu 57 of IGF-1. Molecules with this same type of conformation would work as indirect IGF-1 agonists.

The indirect agonist so identified can be used in a method for treating an agonist disorder wherein an effective amount of the indirect agonist of IGF-1 is administered to a mammal with such a disorder.

Hence, such agonist may be used therapeutically in a pharmaceutical preparation, for example, in clinical

5 trials or commercialized for the agonist disorders as defined herein. Thus, the formulation of the indirect agonist herein can be used to treat any condition that would benefit from treatment with IGF-1, including, for example, diabetes, chronic and acute renal disorders, such as chronic renal insufficiency, necrosis, *etc.*, obesity, hyperinsulinemia, GH-insufficiency, Turner's syndrome, short stature, undesirable symptoms associated with aging such as increasing lean-mass-to-fat ratios, immuno-deficiencies including increasing CD4 counts and increasing immune tolerance, catabolic states associated with wasting, *etc.*, Laron dwarfism, insulin resistance, and so forth.

10 For therapeutic use, the indirect agonist composition herein may be directly administered to the mammal by any suitable technique, including orally, parenterally, intranasally, or intrapulmonarily, and can be administered locally or systemically. The specific route of administration will depend, e.g., on the medical history of the patient, including any perceived or anticipated side or reduced effects using IGF-1, and the disorder to be treated. Examples of parenteral administration include subcutaneous, intramuscular, intravenous, intraarterial, and intraperitoneal administration. Most preferably, the administration is by continuous infusion (using, e.g., minipumps such as osmotic pumps), or by injection (using, e.g.,  
15 intravenous or subcutaneous means). The administration may also be as a single bolus or by slow-release depot formulation. Most preferably, the direct agonist is administered orally or by infusion or injection, at a frequency of, preferably, one-half, once, twice, or three times daily, most preferably daily.

20 The agonist composition to be used in the therapy will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the agonist), the site of delivery of the agonist composition, the method of administration, the scheduling of administration, and other factors known to clinical practitioners. The "effective amount" of agonist for purposes herein is thus determined by such considerations and must be an amount that treats the disorder in question.

25 As a general proposition, the total pharmaceutically effective amount of agonist administered parenterally per dose will be in the range of about 1  $\mu\text{g/kg/day}$  up to about 100  $\text{mg/kg/day}$ , preferably 10  $\mu\text{g/kg/day}$  up to about 10  $\text{mg/kg/day}$ . If given continuously, the agonist is generally administered in doses of about 1  $\mu\text{g/kg/hour}$  up to about 100  $\mu\text{g/kg/hour}$ , either by about 1-4 injections per day or by continuous subcutaneous infusions, for example, using a minipump or a portable infusion pump. An intravenous bag solution may also be employed. The key factor in selecting an appropriate dose is the result obtained as  
30 measured by criteria as are deemed appropriate by the practitioner. If the agonist is administered together with insulin, the latter is used in lower amounts than if used alone, down to amounts which by themselves have little effect on blood glucose, i.e., in amounts of between about 0.1 IU/kg/24 hour to about 0.5 IU/kg/24 hour.

35 For parenteral administration, in one embodiment, the agonist is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.



Generally, the formulation is prepared by contacting the agonist uniformly and intimately with a liquid carrier or a finely divided solid carrier or both. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low-molecular-weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; glycine; amino acids such as glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; nonionic surfactants such as polysorbates, poloxamers, or PEG; and/or neutral salts, e.g., NaCl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, etc.

The agonist is typically formulated individually in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 4.5 to 8. The final formulation, if a liquid, is preferably stored at a temperature of about 2-8°C for up to about four weeks. Alternatively, the formulation can be lyophilized and provided as a powder for reconstitution with water for injection that is stored as described for the liquid formulation.

The agonist to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic agonist compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The agonist ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. The disclosures of all literature and patent citations mentioned herein are expressly incorporated by reference.

## EXAMPLE 1

### Crystallization and Characterization of IGF-1 Crystals

#### Crystallization of IGF-1 and data collection

Recombinant human IGF-1 (rhIGF-1) was obtained as described in the Examples of U.S. Pat. No. 5,723,310 using a polymer/salt combination for phase-forming species and formulated as described in the Examples of U.S. Pat. No. 5,681,814 (acetate, NaCl, polysorbate 20, and benzyl alcohol). Specifically, the initial isolation of IGF-1 from *E. coli* was achieved using aqueous two-phase separation (Hart *et al.*, Bio/Technology, 12: 1113-1117 (1994)), followed by refolding (Hart *et al.*, Biotechnol. Appl. Biochem., 20: 217-232 (1994)), and subsequent chromatographic purification, including large-scale reverse-phase high-performance liquid chromatography (Olson *et al.*, J. Chromatogr., A675: 101-112 (1994)). It was placed in

a vial containing 7 ml of 10 mg/ml rhIGF-1. Prior to crystallization, the IGF-1 was desalted into 0.15 M NaCl and 20 mM sodium acetate (pH 4.5), and diluted to a final concentration of 10 mg/ml. Initially, crystallization trials were set up in the presence of 1 mM of an IGF-1-binding peptide. However, no peptide was ever observed in the crystal, and crystals grown in the absence of the peptide were later shown to be isomorphous to the specimen reported here. A 4- $\mu$ l droplet of the IGF-1 solution was mixed with 5  $\mu$ l of reservoir solution (24% polyethylene glycol 3350 buffered to pH 6.5 with 0.1M sodium cacodylate) and 1  $\mu$ l of 14 mM of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine, which is obtained in a CRYSTAL SCREEN<sup>TM</sup> reagent kit used for crystallization condition screenings and available from Hampton Research, Inc., Laguna Nigel, CA. This solution was allowed to equilibrate via vapor diffusion (Jancarik *et al.*, *supra*) with 1 ml of reservoir solution. Thus, a drop of the mixture was suspended under a plastic cover slip over the reservoir solution. Small crystals with a thin, plate-like morphology appeared within 4-5 days. At this point, 2  $\mu$ l of 100% methyl pentanediol (MPD) (to a final concentration of 20%) was added to the crystallization droplet, and the crystals dissolved overnight. Within 1 week, crystals reappeared and grew to final dimensions of 0.2 mm X 0.1mm X 0.05 mm with noticeably sharper edges. These crystals were used for all subsequent analysis.

Those of skill in the art will appreciate that the aforesaid crystallization conditions can be varied. By varying the crystallization conditions, other crystal forms of IGF-1 may be obtained. Such variations may be used alone or in combination, and include, for example, varying final protein concentrations between about 5 and 35 mg/ml; varying the IGF-1-to-precipitant ratio, varying precipitant concentrations between about 20 and 30% for polyethylene glycol, varying pH ranges between about 5.5 and 7.5, varying the concentration or type of detergent, varying the temperature between about -5 and 30°C, and crystallizing IGF-1 by batch, liquid bridge, or dialysis methods using the above conditions or variations thereof. See McPherson *et al.* (1982), *supra*.

#### Characterization of IGF-1 crystals

A single crystal was transferred from the mother liquor to a cryo-protectant solution consisting of 25% (w/v) polyethylene glycol 3350, 30% MPD, 0.2 M sodium cacodylate pH 6.5, 2.8 mM of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine, and 1 M NaBr. The diffraction was to 1.8 Å. After 30 seconds in this solution, the crystal was flash-cooled by plunging it into liquid nitrogen. The technique of freezing the crystals essentially immortalizes them and produces a much higher quality data set. All subsequent manipulations and x-ray data collection were performed at 100° Kelvin.

A 4-wavelength MAD data set was collected at beamline 9-2 at the Stanford Synchrotron Radiation Laboratory, with the order of the data sets as follows: Br peak ( $\lambda_1$ ), low-energy remote ( $\lambda_2$ ), Br inflection ( $\lambda_3$ ), and high-energy remote ( $\lambda_4$ ). The Br peak and inflection points were estimated from fluorescence scans of the crystal, and the low-energy remote was chosen to be 1.54 angstroms, to maximize the small sulfur anomalous signal at this wavelength while minimizing absorption effects. No inverse beam geometry was used. Data reduction was performed using Denzo and Scalepack (Otwinowski and Minor, Methods in Enzymology, 276: 307-326 (1997)). To determine the most accurate scale and *B*-factors possible, data for all four wavelengths were initially scaled together, assuming no anomalous signal. The scale and *B*-factors determined from this scaling run were then applied to each of the four data sets.

The crystals belong to space group C222<sub>1</sub> with unit cell dimensions or constants of:  $a=31.83$  Å,  $b=71.06$  Å, and  $c=66.00$  Å.  $\alpha=\beta=\gamma=90.000^\circ$ . The asymmetric unit of the crystals contained a monomer of IGF-1 bound to a single detergent molecule, yielding a Matthew's coefficient of  $2.4$  Å<sup>3</sup>/Da, or 48.1% solvent. The solvent content of the crystals was about 55%.

## 5 Structure determination

Initial attempts at determining the structure of IGF-1 by molecular replacement, using either the available NMR models of IGF-1 or the crystal structure of insulin, were unsuccessful. For this reason, the structure was determined *de novo* by Br multiwavelength anomalous dispersion (MAD) (Dauter *et al.*, Acta Crystallogr. D56: 232-237 (2000)).

10 The coordinates of the single-bound bromide were determined by manual inspection of the anomalous and dispersive difference Patterson maps. The hand ambiguity was resolved by phase refinement using the program SHARP (De La Fortelle and Bricogne, Methods in Enzymology, 276: 472-494 (1997)) from Global Phasing Limited, 43 Newton Road, Cambridge CB2 2AL, ENGLAND, followed by examination of anomalous-difference Fourier maps calculated using the  $\lambda 2$  Bijvoet differences. A  
15 cluster of six peaks for one hand of the Br coordinates was consistent with the disulfide structure of insulin (PDB entry: 1ZNI). These six peaks correspond to the six Cys S $\gamma$  atoms in IGF-1; a seventh sulfur (Met S9 S $\delta$ ) was never detected in anomalous-difference Fourier maps, presumably due to its higher temperature factor ( $36.7$  Å<sup>2</sup>). At this point, the six Cys S $\gamma$  positions were included in the phase refinement, with the  $\lambda 1$  data set used as a reference. Throughout the phase refinement, the Br f' was refined for the  $\lambda 1$  data set, f' and f'' were refined for  $\lambda 3$ , and both were kept fixed for data sets  $\lambda 2$  and  $\lambda 4$ ; the f' and f'' values for sulfur were kept fixed at the theoretical values for each wavelength. The small anomalous signal from the sulfur atoms had a modest effect on the phasing statistics, but the resulting electron-density maps showed improved connectivity, especially in the less well ordered regions of IGF-1.

Density modification (solvent flattening and histogram mapping) was performed using DM  
25 (Collaborative Computational Project Number 4, Acta Crystallogr., D50: 760-763 (1994); Cowtan, Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography, 31: 34-38 (1994)), and the resulting electron-density maps were of high quality. Approximately 50% of the structure, corresponding to the three helical regions of IGF-1, was built directly into the experimental electron-density maps using the programs O (Jones *et al.*, Acta Crystallogr., A47: 110-119 (1991)) and QUANTA (version 97.0, MSI, San Diego,  
30 CA). Several rounds of phase combination using Sigmaa (Collaborative Computational Project Number 4, *supra*; Read, Acta Crystallogr., A42: 140-149 (1986)) allowed the remainder of the molecule to be modeled. Atomic positional and restrained *B*-factor refinement utilized the maximum-likelihood target function of CNX (Brünger *et al.*, Acta Crystallogr., D54: 905-921 (1998) and MSI, San Diego, CA), coupled with a "mask"-type bulk solvent correction and anisotropic overall *B*-factor scaling.

35 The final model contains residues 3-34 and 41-64 of IGF-1, one N, N-bis(3-D-gluconamidopropyl)-deoxycholamine molecule, 1 Br<sup>-</sup>, and 50 water molecules. The model was refined against the  $\lambda 3$  data set, since the data statistics demonstrated this data set to be of higher quality than the others. All data from 20- to 1.8-angstrom resolution were included in the refinement, with no application of a sigma cutoff. Secondary structure assignments were made with the program PROMOTIF (Jones *et al.*,  
40 *supra*; Hutchinson and Thornton, Protein Science, 5: 212-220 (1996)).

While the well-ordered positions of IGF-1 were essentially identical using the two sets of phases, the more flexible regions of the molecule showed dramatically improved connectivity upon inclusion of the sulfurs in the phasing. Experimental electron density maps showing the turn region of IGF-1 immediately following the first helix (residues 19, 20, and 21) indicate that using the combined Br and S phases resulted in a much more well-connected map than using just the Br phases alone. At this point, using the Br + S phases, about 50% of the molecule could be traced directly into the experimental maps.

#### Description of the Structure

After several cycles of model building and phase combination, the final model, shown in Figure 2, contains residues 3-34 and 41-64 of IGF-1, a single-bound detergent molecule, and 46 water molecules.

The R factor to 1.8 Å is 23.7%, and the free R factor is 26.9%, with good stereochemistry. The N-terminal B-region corresponds to residues 3-28, the C-region from 29-34, a stretch of poorly ordered residues from 35-40, and the A-region from 42-62. The D-region (63-70) is essentially disordered.

The structure of IGF-1 is similar to insulin (see Figure 3), with a Root-Mean-Squared-Deviation (RMSD) of 3Å over backbone atoms that are conserved between the two molecules. Most of these deviations occur in the flexible regions, and when only the helical regions are considered, the RMSD between alpha-carbon atoms is about 0.47Å. The major difference is the extension of the C-region, for which there is no counterpart in mature insulin, away from the body of the molecule. This loop contains many of the residues that are known to be important for receptor binding.

An extensive alanine-scan mutagenesis study on IGF-1 has shown which residues are important for binding to IGFBP-1 and IGFBP-3 (Dubaque and Lowman, *supra*). The residues that bind to IGFBP-3 are similar to those that bind IGFBP-1, although IGFBP-3 is believed to depend more on backbone interactions and is less severely affected by alanine mutations. There is no one dramatic spot where residues important for IGFBP-1 and IGFBP-3 binding are clustered, and mutations that impair binding are scattered all over the molecule. There appears to be a slight clustering of sites at the N-terminus, with many of these sites being intrinsically hydrophobic.

As shown in Figures 4 and 7, the detergent molecule binds into a small hydrophobic cleft at the base of the B-helix. There are several direct side-chain contacts to the detergent from residues 5, 7, and 10. Despite the overlap of the detergent binding site with a portion of the IGFBP-1/IGFBP-3 binding epitope, the preliminary results suggest, without being limited to any one theory, that the detergent does not inhibit binding of these proteins to IGF-1. The opposite face of the detergent is making a symmetry contact to the opposite face of IGF-1.

As shown in Figure 5, there is only one large crystal packing contact between symmetry-related IGF-1 molecules, which results in a symmetric homodimer. The buried surface area is 1378 Å<sup>2</sup>, which is in the range of physiologically relevant protein-protein interfaces.

Figure 6 shows that the residues known to be important for receptor binding cluster at this dimer interface. Shown are Tyr24, Thr29, Tyr31, and Tyr60. Mutation of these residues results in anywhere from 6-20X loss in affinity for receptor for individual mutations, or 240->1200X loss in affinity for double mutations. Also shown are Phe23 and Phe25, which are interchangeable with Phe24 and Tyr26 of insulin, with no loss of affinity.

### Further Description of the Structure

IGF-1 is composed primarily of three helical segments corresponding to the B-helix (IGF-1 residues 7-18) and two A-helices (IGF-1 residues 43-47 and 54-58) of insulin. The hydrophobic core is essentially identical to that described for the NMR structures of IGF-1, including the three disulfide linkages between Cys 6 and Cys 48, Cys 18 and Cys 61, and Cys 47 and Cys 52, as noted in the references above. Residues 3 through 6 do not form any regular secondary structure, and hence, the structure described herein can be classified as being most similar to the T-form of insulin (Derewenda *et al.*, *Nature*, **338**: 594-596 (1989)). Indeed, when IGF-1 and the T-form of insulin are superimposed on the C $\alpha$  positions of their respective helical segments (IGF-1 residues 8-19, 42-49, and 54-61; insulin residues B9-B20, A1-A8, and A13-A20) the RMSD is only 0.47 angstroms. As in insulin, residues 18-21 at the end of the B-helix form a type II'  $\beta$ -turn, which redirects the backbone from the B-helix into an extended region. Residues 24-27 form a type VIII  $\beta$ -turn to accommodate the C-region, which extends away from the core of IGF-1, and interacts with a symmetry-related molecule. Residues 30-33 form a well-defined type II beta-turn, prominently displaying Tyr 31 at the i+1 position. Residues 35-40 have not been modeled, as the electron density in this region is weak and disconnected. Only the first two residues of the D-region (residues 63 and 64) are ordered in the structure.

The C-region of IGF-1 mediates a two-fold symmetric crystal-packing interaction across the *a*-axis of the unit cell. This interaction buries 689 Å<sup>2</sup> of solvent-accessible surface area from each molecule of IGF-1, or 1378 Å<sup>2</sup> total, and is the largest interface in the crystal. A total of 28 intermolecular contacts of distance 3.6 Å or less are formed via this interface, with the next most extensive crystal packing interaction forming only nine contacts. The core of the interface is dominated by Tyr24 and Pro28 from each monomer, which bury 39 Å<sup>2</sup> and 57 Å<sup>2</sup> of solvent-accessible surface area, respectively. The aromatic ring of Tyr 31, which lies at the tip of the loop at the furthest point from the core of IGF-1, packs against the phenolic rings of Phe 23 and Phe 25 of the symmetry-related molecule. In addition to these hydrophobic interactions, two main-chain hydrogen bonds (Tyr 31 N-Phe 23 O and Ser 34 N-Asp 20 O) are present in the dimer interface. Residues from the D-region (62-64) are also partially sequestered by this dimer formation. Because of these interactions, most of the C-region in the crystal is well-ordered, providing the first high-resolution view of the conformation of this biologically important loop.

Although 72 detergent compounds, including the similar 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulphonate (CHAPS) and 3-((3-cholamidopropyl)dimethylammonio)-2-hydroxypropanesulfonic acid (CHAPSO) detergents, were screened in crystallization trials, only N, N-bis(3-D-gluconamidopropyl)-deoxycholamine yielded crystals. A single molecule of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine interacts with residues, forming a small hydrophobic cleft on one surface of IGF-1 (Leu 5, Phe 16, Val 17, Leu 54, and Leu 57) (Fig. 7A). The preference for N, N-bis(3-D-gluconamidopropyl)-deoxycholamine is explained, without being limited to any one theory, by the absence of an oxygen atom at position C10 in the detergent molecule. This region of the detergent is in close contact with the side chain atoms of residues Leu 5, Leu 54, and Leu 57 in IGF-1. The opposite face of the detergent mediates a symmetry contact with residues Val 11, Leu 14, and Gln 15 of a symmetry-related IGF-1 molecule. Intriguingly, this face of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine also contacts the edge of the dimer interface, with close contacts to Phe 23 and Phe 25 of the same IGF-1 molecule, as

well as Tyr 31 and Gly 32 of the dimeric partner (Fig. 7B). A more detailed analysis indicates that the detergent binds to two patches of binding pockets of IGF-1. One patch has the amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and the second patch has the amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55. Binding is defined by having at least one contact between each listed amino acid residue and the candidate agonist molecule that is less than or equal to 6 angstroms.

### Discussion

The C-region in the IGF-1 crystal structure extends out from the core of the molecule, with residues 30-33 forming a canonical type II beta-turn, and the remainder of the C-region forming a crystallographic dimer with a symmetry-related molecule. Tyr 31 has been implicated as being a critical determinant for IGF-1R binding (Bayne *et al.* (Vol. 264), *supra*; Bayne *et al.* (Vol. 265), *supra*; Cascieri *et al.*, *supra*), and its location at the tip of this extension places it in an ideal location to interact with a receptor molecule. While this region of IGF-1 is not well-defined by NMR data, the conformation of the C-region in the crystal is likely to reflect a prevalent solution conformation. There is evidence of a reverse turn at the tip of the loop and a hinge bending at the loop termini of IGF-2 (Torres *et al.*, *supra*). Thus, while crystal packing forces undoubtedly help stabilize the orientation of this loop, its conformation appears to be consistent with the solution structure of the closely related IGF-2.

The size of the interface formed by the crystallographic dimer is well within the range of buried surface area in known biological complexes (Janin and Chothia, *J. Biol. Chem.*, 264: 16027-16030 (1990)). In addition, this interaction partially excludes from solvent several of the residues known to be important for binding to the IGF-1R, including Phe 23 (69% buried), Tyr 24 (64% buried), Phe 25 (29% buried), and Tyr 31 (38% buried). Other groups have also reported homodimeric interactions of IGF-1 and IGF-2. Laajoki *et al.*, (2000), *supra*, report that at a concentration of 1 mM, an engineered form of IGF-1 (Long-[Arg<sup>3</sup>]IGF-1) partitions into about 20% dimer/80% monomer, a ratio that is in good agreement with the estimate of 3.6 mM K<sub>d</sub>. In their NMR study of IGF-2, Torres *et al.*, *supra*, reported that the amide protons of residues in the C-region were slowly exchanging with solvent, suggesting that IGF-2 forms a homodimer in solution. However, despite the significant amount of surface area that is buried upon dimer formation in the crystal, the affinity of IGF-1 for itself is very weak. In addition, the known binding stoichiometry of one IGF-1 molecule per receptor dimer (De Meyts, *supra*) makes it difficult to rationalize the biological significance of IGF-1 dimerization. In conclusion, the IGF-1 dimer in this crystal form results from the high concentration of IGF-1 in the crystallization experiment, and does not represent a physiologically relevant form of the molecule.

The very low quality of NMR spectroscopic data obtained for IGF-1 at near-neutral pH has been attributed to a combination of self-association and internal mobility that leads to a large variation in resonance line width (Cooke *et al.*, *supra*). As a result, NOESY spectra acquired on IGF-1 contain many broad, overlapped peaks and few sharp well-resolved correlations. NOESY spectra collected for IGF-1 in the presence of an excess of N, N-bis(3-D-gluconamidopropyl)-deoxycholine have a similar appearance. Thus, detergent binding is not sufficient to eliminate the aggregation or inherent flexibility of IGF-1 and does not facilitate characterization of the solution conformation of the protein. Likewise, detergent binding does not alter the aggregation state of IGF-1, as assessed by analytical ultracentrifugation experiments in the

presence of N, N-bis(3-D-gluconamidopropyl)-deoxycholine. This is in contrast to observations in the crystalline state where addition of N, N-bis(3-D-gluconamidopropyl)-deoxycholine leads to a well-packed crystallographic dimer and crystals that diffract to high resolution. Jansson *et al.*, *J. Biol. Chem.*, 273: 24701-24707 (1998) noted that the lack of NMR assignments in the region immediately surrounding Cys 6, which includes Leu 5 and Gly 7, was indicative of the Cys 6-Cys 48 disulfide undergoing intermediate exchange between a cis and trans configuration. The fact that the detergent binds to one face of the B-helix immediately opposite this disulfide suggests, without being limited to any one theory, that it may serve to stabilize this region of the molecule by more complete packing of the hydrophobic cleft. Indeed, in the crystal structure herein, the Cys 6-Cys 48 is clearly in the trans conformation, and there is no evidence of multiple conformations.

### Conclusion

The crystal structure of IGF-1 has been determined using anomalous scattering from the intrinsic sulfur atoms and a Br<sup>-</sup> ion bound at a fortuitous halide-binding site. The structure is very similar to insulin, with the only major difference being the C-region, which protrudes from the body of the protein and mediates a homodimeric interaction. The amount of buried surface area is consistent with the fact that at neutral pH, IGF-1 undergoes self-association in a concentration-dependent manner. In addition, several residues that are important for receptor binding are found at this dimer interface, suggesting, without being limited to any one theory, that effects on receptor binding by mutation of these residues may be a result of disruption of the dimer, rather than direct contact with the receptor surface.

## EXAMPLE 2

### Diffusion-based measurement of detergent binding

NMR-derived diffusion measurements were used to estimate the  $K_d$  for the interaction between IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholine. Samples were prepared in 50 mM phosphate buffer in D<sub>2</sub>O, pH 6.5 (uncorrected meter reading), and contained: 1.0 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholine + 0.5 mM IGF-1; 0.5 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholine + 0.25 mM IGF-1; 0.25 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholine + 0.125 mM IGF-1; or N, N-bis(3-D-gluconamidopropyl)-deoxycholine only (1.0, 0.5, or 0.25 mM). All spectra were acquired at 40°C on a Bruker AVANCE 500<sup>TM</sup> spectrometer (Bruker Analytik GmbH) equipped with a 5-mm triple-axis gradient, triple-resonance probe. Diffusion measurements were made with a bipolar pulse pair method with  $\delta = 5$  ms,  $\tau = 2$  ms, and  $\Delta = 25$  or 40 ms for N, N-bis(3-D-gluconamidopropyl)-deoxycholine alone or N, N-bis(3-D-gluconamidopropyl)-deoxycholine + IGF-1, respectively (Wu *et al.*, *J. Magn. Reson., Ser. A* 115: 260-264 (1995)). Spectra were collected with 128 to 1024 transients as the z-gradient strength was increased from 0.009 to 0.45 T•m<sup>-1</sup> in 18 equal increments; measurements were made at least twice on each sample. Spectra were processed and peak heights extracted with the program FELIX (v98.0, MSI, San Diego). Diffusion constants, proportion of bound detergent, and resulting  $K_d$  were extracted as described by Fejzo *et al.*, *Chemistry & Biology*, 6: 755-769 (1999). Spectra were also collected on samples containing 1.0 mM 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate, a zwitterionic detergent used for membrane solubilization, and 1.0 mM 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate + 0.5 mM IGF-1. Two-dimensional NOESY spectra (Jeener *et*

*al.*, *J. Chem. Phys.*, **71**: 4546-4553 (1979)) were collected on a 0.5-mM sample of IGF-1 in the presence or absence of 1.0 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholine with a mixing time of 100 ms.

#### IGF-1 phage ELISA

*E. coli* cells (XL1-Blue, Stratagene) freshly transformed with the phage vector pIGF-g3 displaying human IGF-1 as described in Dubaquié and Lowman, *supra*, were grown overnight in 5 ml of 2YT medium (Sambrook *et al.*, *Molecular Cloning: A Laboratory Handbook* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)). The phage particles displaying IGF-1 were titered against IGFBP-1 and IGFBP-3 to obtain a 500-1000-fold dilution for preincubation with serial dilutions of the detergents and binding protein standards for 45 minutes. Microwell clear polystyrene immunoplates with a MAXISORP™ surface (Nunc, Denmark) were coated with IGFBP-1 or IGFBP-3 protein overnight at 4°C (50 µl at 3 µg/mL in 50 mM carbonate buffer, pH 9.6), blocked with 0.5% TWEEN® 20 polyoxyethylene sorbitan monolaurate (Atlas Chemical Co.), and PBS and washed eight times with PBS and 0.05% TWEEN® 20 polyoxyethylene sorbitan monolaurate. The samples were added to the plates for 30 minutes. Plates were washed eight times with PBS and 0.05% TWEEN® 20 polyoxyethylene sorbitan monolaurate, incubated with 50 µL of 1:10,000 horseradish peroxidase/anti-M13 antibody conjugate (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS and 0.5% BSA for 30 minutes, and then washed eight times with PBS and 0.05% TWEEN® 20 polyoxyethylene sorbitan monolaurate and two times with PBS. Plates were developed using a tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD), stopped with 1.0 H<sub>3</sub>PO<sub>4</sub>, and read spectrophotometrically at 450 nm.

#### Sedimentation equilibrium analysis

The self-association of IGF-1 was determined by sedimentation equilibrium analysis. The experiments were conducted at 20°C in an OPTIMA™ XL-A/XL-I analytical ultracentrifuge (Beckman Coulter, Inc.). The samples were prepared in 0.1 M citrate buffer, pH 6.5, 75 mM NaCl with a loading concentration from 1 mM to 0.01 mM. The concentration gradients were measured at rotor speeds of 25000 and 30000 rpm at 280 nm or 285 nm using a scanning absorption optical system. The attainment of an equilibrium state was verified by comparing successive scans after approximately 16 hours. The partial specific volume of IGF-1 was calculated from its amino acid composition. The data were fit as a single ideal species or the ideal dimer self-association models using a non-linear least-squares fitting program, NONLIN (Johnson *et al.*, *Biophys. J.*, **36**: 578-588 (1981)). The association constants were determined from the best-fit values of the model, returned by non-linear least-squares regression.

#### Results:

##### N, N-bis(3-D-gluconamidopropyl)-deoxycholine binds to IGF-1 in solution.

The affinity of IGF-1 for 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate and N, N-bis(3-D-gluconamidopropyl)-deoxycholine was ascertained using solution-NMR methods. The chemical shift changes observed during a titration of N, N-bis(3-D-gluconamidopropyl)-deoxycholine into a 0.5 mM IGF-1 solution suggested that the affinity was submillimolar and not easily measurable from such data. Instead, diffusion measurements were made on samples at varying IGF-1 concentrations containing 2 molar equivalents of detergent and also on several samples of detergent alone (the detergent concentration was always less than the critical micelle concentration of 1.4 mM for N, N-bis(3-D-gluconamidopropyl)-deoxycholine and 14 mM for 3-((3-cholamidopropyl) dimethylammonio)-1-propane



sulphonate). The decrease in diffusion constant of the detergent in the presence of the protein can be used to estimate the proportion of detergent bound to the protein (Fejzo *et al.*, *supra*). Since the total concentration of detergent and protein is known, a value of the dissociation constant can be determined. At the three protein concentrations studied (0.5 mM, 0.25 mM, and 0.125 mM),  $K_d$  values of 220, 440, and 430  $\mu$ M were obtained, respectively. This technique has routinely been applied to small molecules (several hundred Daltons molecular weight or less) binding to large proteins. In this particular case, the ligand is relatively large (862 Da) and the protein is relatively small (7648 Da); hence, the differential decrease in diffusion constant on binding is small. This increases the uncertainty with which the dissociation constant can be measured. Given this, the data described above suggest that the  $K_d$  for the interaction between N, N-bis(3-D-gluconamidopropyl)-deoxycholine and IGF-1 is  $300 \pm 150 \mu$ M. A similar analysis of the (3-(3-cholamidopropyl) dimethylammonio)-1-propane sulphonate diffusion data suggests that that  $K_d$  in this case is greater than 3 mM.

#### N, N-bis(3-D-gluconamidopropyl)-deoxycholine blocks IGFBP-1 and IGFBP-3 binding.

To examine the binding epitope of N, N-bis(3-D-gluconamidopropyl)-deoxycholine on IGF-1, the detergent was preincubated with IGF-1 expressed on bacteriophage particles, and the level of residual binding to IGFBP-1 and IGFBP-3 was measured in a plate-based assay (ELISA). As a control, soluble IGFBP-1 was also tested. As shown in Figure 8, N, N-bis(3-D-gluconamidopropyl)-deoxycholine inhibited IGF-1 on phage from binding to IGFBP-1 and IGFBP-3 with  $IC_{50}$  values of  $480 \pm 170 \mu$ M and  $275 \pm 152 \mu$ M, respectively. These numbers must be interpreted conservatively, however, since the critical micelle concentration of N, N-bis(3-D-gluconamidopropyl)-deoxycholine (1.4 mM) presents an upper limit on the curve in Figure 8. In contrast to the effect of N, N-bis(3-D-gluconamidopropyl)-deoxycholine, the closely related detergent 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate did not show any inhibition of binding at any of the concentrations tested up to 1 mM. Despite the limitations of the experiment, the  $IC_{50}$  values obtained for N, N-bis(3-D-gluconamidopropyl)-deoxycholine are in good agreement with the NMR-based estimate of a  $K_d$  of  $\sim 300 \mu$ M for the N, N-bis(3-D-gluconamidopropyl)-deoxycholine-IGF-1 interaction.

#### Self-association of IGF-1.

The sedimentation equilibrium data show that IGF-1 undergoes self-association in solution. The average molecular weight increased with increasing protein concentration from 0.01 mM to 1 mM. The average molecular weight at the highest concentration studied (1 mM) is about 37% higher than the monomer molecular weight (10.4 KDa at 1 mM versus 7.6 KDa monomer molecular weight). At concentrations below 0.05 mM, no self-association was observed, and IGF-1 exists only as a monomer in solution at neutral pH. If it is assumed that the higher-molecular-weight species are IGF-1 dimers, the sedimentation data can be fit as a monomer-dimer model with a  $K_d$  of  $3.6 \pm 1.0$  mM (Figure 9).

#### Discussion

Several studies have identified residues in IGF-1 that are important for IGFBP binding (Clemmons *et al.*, *Endocrinology*, **131**: 890-895 (1992); Dubaquié and Lowman, *supra*; Jansson *et al.*, *supra*; Oh *et al.*, (1993), *supra*; Lowman *et al.*, (1998), *supra*; and Dubaquié *et al.*, *Endocrinology*, **142**: 165-173 (2001)). Dubaquié and Lowman, *supra*, identified two distinct patches on IGF-1 that interact with IGFBP-1 and IGFBP-3. Patch I consists of Glu 7, Leu 10, Val 11, Leu 14, Phe 25, Ile 43, and Val 44, while patch 2

consists of Glu 3, Thr 4, Leu 5, Phe 16, Val 17, and Leu 54. In the crystal structure of IGF-1, these two patches are involved in detergent-mediated crystal packing contacts. (Specifically, Patch 1 of the crystal structure of IGF-1 consists of amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and Patch 2 of the crystal structure of IGF-1 consists of amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55, wherein binding occurs if there is at least one contact between each listed amino acid residue and the candidate agonist molecule that is less than or equal to 6 angstroms.)

The overlap of the detergent binding site with the IGFBP interaction surfaces is entirely consistent with the observation herein that N, N-bis(3-D-gluconamidopropyl)-deoxycholine blocks IGFBP-1 and IGFBP-3 binding. In contrast, N, N-bis(3-D-gluconamidopropyl)-deoxycholine does not inhibit IGF-1R-mediated signaling in a cell-based receptor activation assay. These results are consistent with prior studies that demonstrated different binding epitopes on IGF-1 for receptor and IGFBP interactions (Bayne *et al.*, *supra*, (Vol. 264); Bayne *et al.*, *supra*, (Vol. 265); Cascieri *et al.*, *supra*). The identification of N, N-bis(3-D-gluconamidopropyl)-deoxycholine as an inhibitor of IGFBP interactions allows the ability to develop small-molecule drugs or peptidomimetics that disrupt the IGF-1/IGFBP complex *in vivo*, thereby releasing receptor-active IGF-1 from the systemic, inactive pool. Such drugs include orally bioavailable therapy for metabolic disease such as diabetes.

Recently, Zeslawski *et al.* (EMBO J., 20: 3638-3644 (2001)) published the crystal structure of IGF-1 in complex with the N-terminal domain of IGFBP-5. The structure of that complex is entirely consistent with the model of detergent inhibition of IGFBP binding presented herein, and also disclosed by Vajdos *et al.*, Biochemistry, 40: 11022-11029 (2001). The NMR determination of a complex of IGF-1 bound to a phage-derived IGF-1 antagonist peptide designated IGF-F1-1 (RNCFESVAALRRRCMYG (SEQ ID NO:4)), in comparison with other IGF-1 crystal structures, shows that, without limitation to any one theory, a portion of the A-chain (helix III) is mobile in solution, and adopts slightly different conformations when bound to different ligands (detergent, peptide, binding protein).

The complex between peptide IGF-F1-1 and IGF-1 was determined from NMR spectroscopy data collected at 600 and 800 MHz. IGF-1 uniformly labeled with  $^{13}\text{C}$  and  $^{15}\text{N}$  was prepared using the scheme outlined by Reilly and Fairbrother, J. Biomol. NMR, 4: 459-462 (1994) and purified according to the protocol in Vajdos *et al.*, *supra*. A slight molar excess of unlabeled IGF-F1-1 was mixed with a 1.5 mM solution of  $^{13}\text{C}/^{15}\text{N}$  IGF-1 and  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  NMR resonances assigned from double- and triple-resonance NMR experiments as described by Cavanagh *et al.* in Protein NMR Spectroscopy, Principles and Practice (Academic Press: New York, 1996). Distance restraints within IGF-1 were identified from  $^{13}\text{C}$ -edited NOESY HSQC spectra and  $^{15}\text{N}$ -edited NOESY HSQC spectra (Cavanagh *et al.*, *supra*).

Intermolecular restraints between IGF-1 and the peptide were obtained from an  $\omega$ 1-filtered,  $\omega$ 2-edited  $^{13}\text{C}$  HSQC-NOESY spectrum (Lee *et al.*, FEBS Lett., 350: 87-90 (1994)). Intrapeptide distance restraints were obtained from a 2-D  $^{13}\text{C}$ -filtered NOESY spectrum. In addition,  $\phi$  dihedral angle restraints were obtained from an HNHA spectrum (Cavanagh *et al.*, *supra*), and  $\chi$ 1 restraints were derived from HNHB and short-mixing-time TOCSY spectra (Clare *et al.*, J. Biomolec. NMR, 1: 13-22 (1991)). Additional  $\phi, \psi$  restraints were obtained from an analysis of the  $\text{H}^\alpha$ , N,  $\text{C}^\alpha$ ,  $\text{C}^\beta$ , and CO chemical shifts using the program TALOS (Cornilescu *et al.*, J. Biomol. NMR, 13: 289-302 (1999)).

In total, 899 distance restraints (779 intra-IGF-1; 33 intra-peptide; 87 intermolecular), 16 hydrogen bond restraints in helix I, and 138 dihedral angle restraints (71  $\phi$ ; 44  $\psi$ ; 23  $\chi$ 1) were used to generate an ensemble of structures using a torsion-angle dynamics protocol with the computer program CNX (Accelrys Inc., San Diego). The structure of IGF-1 was well defined for the B-region (residues 2 – 25) and the A-region (residues 41-63) with a mean RMSD from the mean structure for backbone heavy atoms of  $0.32 \pm 0.06$  Å. The C-region (26-40) and the D-region (62-70) were not well defined by the available data. The 20 structures of lowest restraint violation energy had good backbone stereochemistry (80% of residues in the most favored region of  $\phi/\psi$  space with none in disallowed regions) and contained few violations of the experimental restraints (mean maximum distance restraint violation  $0.09 \pm 0.02$  Å). IGF-F1-1 adopts a conformation very similar to that determined for the peptide by itself in solution. The conformation of IGF-1 contains three helices (residues 7-18, 43-49, and 54-60) and is similar to that seen at lower resolution in previous NMR studies of uncomplexed IGF-1 (see *e.g.* Cooke *et al.*, *supra*; Sato *et al.*, *supra*; and Laajoki *et al.*, *supra*).

Fig. 10 shows the comparison for the detergent and phage peptide complexes. Specifically, Fig. 10A shows a ribbon diagram of a complex of IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine), and Fig. 10B shows a complex of IGF-1 bound to the phage-derived peptide IGF-F1-1. The B-region (helix I) adopts a very similar conformation in both complexes. The C-loop is only partially ordered in the detergent complex, and ill defined in the peptide complex. Ligand-induced differences are observed for the A-region of IGF-1 (Helix III), at both the backbone (residues 52-60) and side chain (leucine 54 and 57) level. Without limitation to any one theory, maleability in this A-region area is believed to be what allows IGF-1 to bind to so many proteins (six IGFBPs and three receptors).

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the objectives of the present invention.

## APPENDIX 1

REMARK 3 CRYSTAL STRUCTURE OF IGF-1 SOLVED USING MAD  
REMARK 3 REFINEMENT.

5 REMARK 3 PROGRAM : X-PLOR(online) 98.1  
REMARK 3 AUTHORS : BRUNGER  
REMARK 3  
REMARK 3 DATA USED IN REFINEMENT.

10 REMARK 3 RESOLUTION RANGE HIGH (ANGSTROMS) : 1.80  
REMARK 3 RESOLUTION RANGE LOW (ANGSTROMS) : 20.00  
REMARK 3 DATA CUTOFF (SIGMA(F)) : 0.2  
REMARK 3 DATA CUTOFF HIGH (ABS(F)) : 10000000.00  
REMARK 3 DATA CUTOFF LOW (ABS(F)) : 0.001000  
REMARK 3 COMPLETENESS (WORKING+TEST) (%) : 94.8

15 REMARK 3 NUMBER OF REFLECTIONS : 6870  
REMARK 3  
REMARK 3 FIT TO DATA USED IN REFINEMENT.

20 REMARK 3 CROSS-VALIDATION METHOD : THROUGHOUT  
REMARK 3 FREE R VALUE TEST SET SELECTION : RANDOM  
REMARK 3 R VALUE (WORKING SET) : 0.237  
REMARK 3 FREE R VALUE : 0.269  
REMARK 3 FREE R VALUE TEST SET SIZE (%) : 5.6  
REMARK 3 FREE R VALUE TEST SET COUNT : 382  
REMARK 3 ESTIMATED ERROR OF FREE R VALUE : 0.014

25 REMARK 3  
REMARK 3 FIT IN THE HIGHEST RESOLUTION BIN.

30 REMARK 3 TOTAL NUMBER OF BINS USED : 6  
REMARK 3 BIN RESOLUTION RANGE HIGH (A) : 1.80  
REMARK 3 BIN RESOLUTION RANGE LOW (A) : 1.91  
REMARK 3 BIN COMPLETENESS (WORKING+TEST) (%) : 74.4  
REMARK 3 REFLECTIONS IN BIN (WORKING SET) : 826  
REMARK 3 BIN R VALUE (WORKING SET) : 0.343  
REMARK 3 BIN FREE R VALUE : 0.439  
REMARK 3 BIN FREE R VALUE TEST SET SIZE (%) : 5.5

35 REMARK 3 BIN FREE R VALUE TEST SET COUNT : 48  
REMARK 3 ESTIMATED ERROR OF BIN FREE R VALUE : 0.063  
REMARK 3  
REMARK 3 NUMBER OF NON-HYDROGEN ATOMS USED IN REFINEMENT.

40 REMARK 3 PROTEIN ATOMS : 475  
REMARK 3 NUCLEIC ACID ATOMS : 0

REMARK 3 HETEROGEN ATOMS : 62  
REMARK 3 SOLVENT ATOMS : 102  
REMARK 3  
REMARK 3 B VALUES.  
5 REMARK 3 FROM WILSON PLOT (A\*\*2) : 25.1  
REMARK 3 MEAN B VALUE (OVERALL, A\*\*2) : 33.8  
REMARK 3 OVERALL ANISOTROPIC B VALUE.  
REMARK 3 B11 (A\*\*2) : 0.00  
REMARK 3 B22 (A\*\*2) : 0.00  
10 REMARK 3 B33 (A\*\*2) : 0.00  
REMARK 3 B12 (A\*\*2) : 0.00  
REMARK 3 B13 (A\*\*2) : 0.00  
REMARK 3 B23 (A\*\*2) : 0.00  
REMARK 3  
15 REMARK 3 ESTIMATED COORDINATE ERROR.  
REMARK 3 ESD FROM LUZZATI PLOT (A) : 0.23  
REMARK 3 ESD FROM SIGMAA (A) : 0.16  
REMARK 3 LOW RESOLUTION CUTOFF (A) : 20.00  
REMARK 3  
20 REMARK 3 CROSS-VALIDATED ESTIMATED COORDINATE ERROR.  
REMARK 3 ESD FROM C-V LUZZATI PLOT (A) : 0.28  
REMARK 3 ESD FROM C-V SIGMAA (A) : 0.18  
REMARK 3  
REMARK 3 RMS DEVIATIONS FROM IDEAL VALUES.  
25 REMARK 3 BOND LENGTHS (A) : 0.020  
REMARK 3 BOND ANGLES (DEGREES) : 2.0  
REMARK 3 DIHEDRAL ANGLES (DEGREES) : 23.2  
REMARK 3 IMPROPER ANGLES (DEGREES) : 1.09  
REMARK 3  
30 REMARK 3 ISOTROPIC THERMAL MODEL : RESTRAINED  
REMARK 3  
REMARK 3 ISOTROPIC THERMAL FACTOR RESTRAINTS. RMS SIGMA  
REMARK 3 MAIN-CHAIN BOND (A\*\*2) : 4.33 ; 4.00  
REMARK 3 MAIN-CHAIN ANGLE (A\*\*2) : 6.46 ; 4.00  
35 REMARK 3 SIDE-CHAIN BOND (A\*\*2) : 5.41 ; 8.50  
REMARK 3 SIDE-CHAIN ANGLE (A\*\*2) : 8.14 ; 9.00  
REMARK 3  
REMARK 3 NCS MODEL : NONE  
REMARK 3  
40 REMARK 3 NCS RESTRAINTS. RMS SIGMA/WEIGHT

REMARK 3 GROUP 1 POSITIONAL (A): NULL ; NULL  
 REMARK 3 GROUP 1 B-FACTOR (A\*\*2): NULL ; NULL  
 REMARK 3  
 REMARK 3 PARAMETER FILE 1 : MSI\_XPLOR\_TOPPAR/protein\_rep.param  
 5 REMARK 3 PARAMETER FILE 2 : ../parameter.element  
 REMARK 3 PARAMETER FILE 3 : ../cyc.par  
 REMARK 3 PARAMETER FILE 4 : ../g2mz/param19.sol  
 REMARK 3 TOPOLOGY FILE 1 : /usr/prop/msi/980/data/xplor/toppar/tophcsdx.pro  
 REMARK 3 TOPOLOGY FILE 2 : ../topology.element  
 10 REMARK 3 TOPOLOGY FILE 3 : ../cyc.top  
 REMARK 3 TOPOLOGY FILE 4 : ../g2mz/toph19.sol  
 REMARK 3  
 REMARK 3 OTHER REFINEMENT REMARKS: BULK SOLVENT MODEL USED  
 SEQRES 1 A 100 GLU THR LEU CYS GLY ALA GLU LEU VAL ASP ALA LEU GLN  
 15 SEQRES 2 A 100 PHE VAL CYS GLY ASP ARG GLY PHE TYR PHE ASN LYS PRO  
 SEQRES 3 A 100 THR GLY TYR GLY SER SER THR GLY ILE VAL ASP GLU CYS  
 SEQRES 4 A 100 CYS PHE ARG SER CYS ASP LEU ARG ARG LEU GLU MET TYR  
 SEQRES 5 A 100 CYS ALA PRO LEU HOH HOH HOH HOH HOH HOH HOH HOH  
 SEQRES 6 A 100 HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH  
 20 SEQRES 7 A 100 HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH  
 SEQRES 8 A 100 HOH HOH HOH HOH HOH HOH HOH HOH HOH  
 SEQRES 1 B 3 CYC BR HOH  
 SSBOND 1 CYS A 6 CYS A 48  
 SSBOND 2 CYS A 18 CYS A 61  
 25 SSBOND 3 CYS A 47 CYS A 52  
 CRYST1 31.830 71.074 66.014 90.00 90.00 90.00 C 2 2 21 16  
 ORIGX1 1.000000 0.000000 0.000000 0.000000  
 ORIGX2 0.000000 1.000000 0.000000 0.000000  
 ORIGX3 0.000000 0.000000 1.000000 0.000000  
 30 SCALE1 0.031417 0.000000 0.000000 0.000000  
 SCALE2 0.000000 0.014070 0.000000 0.000000  
 SCALE3 0.000000 0.000000 0.015148 0.000000  
 REMARK FILENAME="final.pdb"  
 REMARK r= 0.237371 free\_r= 0.26887  
 35 REMARK DATE:Jan-30-01 14:51:37 created by user: mhu  
 ATOM 1 CB GLU A 3 20.808 24.058 21.698 1.00 46.92  
 ATOM 2 CG GLU A 3 19.515 23.283 21.499 1.00 61.66  
 ATOM 3 CD GLU A 3 18.281 24.029 22.025 1.00 70.74  
 ATOM 4 OE1 GLU A 3 18.254 24.408 23.221 1.00 74.11  
 40 ATOM 5 OE2 GLU A 3 17.332 24.237 21.238 1.00 75.50

	ATOM	6	C	GLU	A	3	22.417	22.169	21.324	1.00	36.23
	ATOM	7	O	GLU	A	3	21.616	21.223	21.375	1.00	38.90
	ATOM	8	N	GLU	A	3	21.623	23.389	19.405	1.00	38.88
	ATOM	9	CA	GLU	A	3	21.994	23.550	20.854	1.00	40.66
5	ATOM	10	N	THR	A	4	23.696	22.040	21.647	1.00	31.38
	ATOM	11	CA	THR	A	4	24.213	20.754	22.059	1.00	24.88
	ATOM	12	CB	THR	A	4	25.301	20.217	21.051	1.00	23.86
	ATOM	13	OG1	THR	A	4	26.426	21.106	21.070	1.00	30.08
	ATOM	14	CG2	THR	A	4	24.786	20.115	19.645	1.00	25.92
10	ATOM	15	C	THR	A	4	24.825	20.983	23.441	1.00	20.71
	ATOM	16	O	THR	A	4	24.715	22.065	24.036	1.00	19.97
	ATOM	17	N	LEU	A	5	25.435	19.949	23.986	1.00	19.32
	ATOM	18	CA	LEU	A	5	25.976	20.054	25.319	1.00	15.87
	ATOM	19	CB	LEU	A	5	25.300	19.036	26.254	1.00	22.19
15	ATOM	20	CG	LEU	A	5	23.859	19.367	26.714	1.00	28.27
	ATOM	21	CD1	LEU	A	5	23.229	18.123	27.479	1.00	23.45
	ATOM	22	CD2	LEU	A	5	23.916	20.612	27.594	1.00	25.44
	ATOM	23	C	LEU	A	5	27.472	19.662	25.252	1.00	19.16
	ATOM	24	O	LEU	A	5	27.742	18.527	24.980	1.00	20.58
20	ATOM	25	N	CYS	A	6	28.352	20.591	25.572	1.00	19.74
	ATOM	26	CA	CYS	A	6	29.809	20.320	25.520	1.00	21.84
	ATOM	27	C	CYS	A	6	30.485	20.754	26.790	1.00	16.04
	ATOM	28	O	CYS	A	6	29.990	21.602	27.535	1.00	18.05
	ATOM	29	CB	CYS	A	6	30.448	21.077	24.361	1.00	22.37
25	ATOM	30	SG	CYS	A	6	29.753	20.589	22.733	1.00	32.91
	ATOM	31	N	GLY	A	7	31.685	20.185	27.039	1.00	19.09
	ATOM	32	CA	GLY	A	7	32.397	20.613	28.217	1.00	17.15
	ATOM	33	C	GLY	A	7	31.698	20.668	29.536	1.00	18.61
	ATOM	34	O	GLY	A	7	31.069	19.683	29.965	1.00	15.50
30	ATOM	35	N	ALA	A	8	31.835	21.779	30.240	1.00	13.90
	ATOM	36	CA	ALA	A	8	31.276	21.897	31.553	1.00	15.72
	ATOM	37	CB	ALA	A	8	31.630	23.239	32.150	1.00	21.73
	ATOM	38	C	ALA	A	8	29.715	21.774	31.498	1.00	14.12
	ATOM	39	O	ALA	A	8	29.116	21.316	32.469	1.00	14.45
35	ATOM	40	N	GLU	A	9	29.145	22.263	30.412	1.00	15.00
	ATOM	41	CA	GLU	A	9	27.653	22.198	30.355	1.00	21.86
	ATOM	42	CB	GLU	A	9	27.118	22.975	29.140	1.00	23.00
	ATOM	43	CG	GLU	A	9	27.116	24.481	29.346	1.00	33.94
	ATOM	44	CD	GLU	A	9	26.424	25.279	28.204	1.00	53.09
40	ATOM	45	OE1	GLU	A	9	25.790	24.700	27.260	1.00	50.65

	ATOM	46	OE2	GLU	A	9	26.521	26.528	28.270	1.00	63.42
	ATOM	47	C	GLU	A	9	27.200	20.732	30.278	1.00	19.13
	ATOM	48	O	GLU	A	9	26.180	20.376	30.831	1.00	15.61
	ATOM	49	N	LEU	A	10	27.974	19.902	29.589	1.00	16.50
5	ATOM	50	CA	LEU	A	10	27.688	18.488	29.435	1.00	19.32
	ATOM	51	CB	LEU	A	10	28.637	17.845	28.384	1.00	16.05
	ATOM	52	CG	LEU	A	10	28.552	16.296	28.272	1.00	18.02
	ATOM	53	CD1	LEU	A	10	27.080	15.875	27.864	1.00	17.22
	ATOM	54	CD2	LEU	A	10	29.459	15.858	27.153	1.00	17.33
10	ATOM	55	C	LEU	A	10	27.850	17.812	30.788	1.00	20.39
	ATOM	56	O	LEU	A	10	27.030	16.968	31.173	1.00	13.49
	ATOM	57	N	VAL	A	11	28.924	18.126	31.531	1.00	16.73
	ATOM	58	CA	VAL	A	11	29.089	17.550	32.838	1.00	13.49
	ATOM	59	CB	VAL	A	11	30.518	17.917	33.453	1.00	16.29
15	ATOM	60	CG1	VAL	A	11	30.636	17.370	34.882	1.00	19.22
	ATOM	61	CG2	VAL	A	11	31.603	17.350	32.528	1.00	17.93
	ATOM	62	C	VAL	A	11	28.006	18.025	33.824	1.00	13.78
	ATOM	63	O	VAL	A	11	27.532	17.272	34.668	1.00	11.61
	ATOM	64	N	ASP	A	12	27.599	19.280	33.717	1.00	14.34
20	ATOM	65	CA	ASP	A	12	26.573	19.795	34.651	1.00	15.14
	ATOM	66	CB	ASP	A	12	26.166	21.243	34.290	1.00	14.66
	ATOM	67	CG	ASP	A	12	26.960	22.337	34.970	1.00	27.52
	ATOM	68	OD1	ASP	A	12	27.448	22.152	36.103	1.00	29.53
	ATOM	69	OD2	ASP	A	12	27.031	23.422	34.314	1.00	31.00
25	ATOM	70	C	ASP	A	12	25.275	18.941	34.366	1.00	11.41
	ATOM	71	O	ASP	A	12	24.564	18.555	35.313	1.00	14.02
	ATOM	72	N	ALA	A	13	25.006	18.744	33.115	1.00	16.37
	ATOM	73	CA	ALA	A	13	23.761	18.002	32.720	1.00	18.28
	ATOM	74	CB	ALA	A	13	23.558	18.014	31.189	1.00	15.65
30	ATOM	75	C	ALA	A	13	23.803	16.590	33.235	1.00	17.95
	ATOM	76	O	ALA	A	13	22.833	16.073	33.788	1.00	15.70
	ATOM	77	N	LEU	A	14	24.954	15.920	33.065	1.00	11.92
	ATOM	78	CA	LEU	A	14	25.077	14.560	33.611	1.00	15.01
	ATOM	79	CB	LEU	A	14	26.443	13.965	33.164	1.00	13.58
35	ATOM	80	CG	LEU	A	14	26.457	13.638	31.695	1.00	15.59
	ATOM	81	CD1	LEU	A	14	27.939	13.345	31.250	1.00	17.99
	ATOM	82	CD2	LEU	A	14	25.601	12.370	31.414	1.00	22.05
	ATOM	83	C	LEU	A	14	24.944	14.495	35.098	1.00	15.56
	ATOM	84	O	LEU	A	14	24.341	13.550	35.626	1.00	18.96
40	ATOM	85	N	GLN	A	15	25.591	15.431	35.831	1.00	12.18



	ATOM	86	CA	GLN A 15	25.494	15.426	37.273	1.00	14.03
	ATOM	87	CB	GLN A 15	26.379	16.526	37.916	1.00	17.47
	ATOM	88	CG	GLN A 15	27.917	16.185	37.673	1.00	23.70
	ATOM	89	CD	GLN A 15	28.863	16.851	38.656	1.00	32.76
5	ATOM	90	OE1	GLN A 15	29.187	18.020	38.510	1.00	28.85
	ATOM	91	NE2	GLN A 15	29.314	16.095	39.658	1.00	29.69
	ATOM	92	C	GLN A 15	24.061	15.631	37.745	1.00	18.29
	ATOM	93	O	GLN A 15	23.684	15.090	38.729	1.00	18.76
	ATOM	94	N	PHE A 16	23.297	16.400	37.012	1.00	18.23
10	ATOM	95	CA	PHE A 16	21.916	16.692	37.438	1.00	17.04
	ATOM	96	CB	PHE A 16	21.438	17.935	36.706	1.00	18.66
	ATOM	97	CG	PHE A 16	20.060	18.334	37.110	1.00	18.49
	ATOM	98	CD1	PHE A 16	19.874	18.960	38.310	1.00	23.45
	ATOM	99	CD2	PHE A 16	18.959	17.947	36.339	1.00	21.34
15	ATOM	100	CE1	PHE A 16	18.558	19.216	38.788	1.00	27.25
	ATOM	101	CE2	PHE A 16	17.646	18.190	36.789	1.00	21.50
	ATOM	102	CZ	PHE A 16	17.457	18.829	38.020	1.00	24.95
	ATOM	103	C	PHE A 16	21.018	15.479	37.110	1.00	15.52
	ATOM	104	O	PHE A 16	20.248	15.013	37.971	1.00	20.37
20	ATOM	105	N	VAL A 17	21.160	14.923	35.916	1.00	16.12
	ATOM	106	CA	VAL A 17	20.338	13.761	35.490	1.00	17.94
	ATOM	107	CB	VAL A 17	20.392	13.598	33.932	1.00	21.28
	ATOM	108	CG1	VAL A 17	19.831	12.219	33.456	1.00	25.51
	ATOM	109	CG2	VAL A 17	19.619	14.737	33.295	1.00	22.15
25	ATOM	110	C	VAL A 17	20.720	12.454	36.182	1.00	21.98
	ATOM	111	O	VAL A 17	19.843	11.655	36.556	1.00	24.05
	ATOM	112	N	CYS A 18	22.015	12.222	36.411	1.00	17.42
	ATOM	113	CA	CYS A 18	22.430	10.964	37.039	1.00	20.54
	ATOM	114	C	CYS A 18	22.420	10.998	38.579	1.00	26.67
30	ATOM	115	O	CYS A 18	22.386	9.967	39.239	1.00	24.96
	ATOM	116	CB	CYS A 18	23.841	10.565	36.505	1.00	16.00
	ATOM	117	SG	CYS A 18	23.947	10.463	34.717	1.00	29.49
	ATOM	118	N	GLY A 19	22.462	12.198	39.147	1.00	32.94
	ATOM	119	CA	GLY A 19	22.464	12.366	40.595	1.00	31.76
35	ATOM	120	C	GLY A 19	23.563	11.580	41.263	1.00	37.69
	ATOM	121	O	GLY A 19	24.730	11.631	40.869	1.00	40.61
	ATOM	122	N	ASP A 20	23.186	10.815	42.276	1.00	39.20
	ATOM	123	CA	ASP A 20	24.150	10.009	42.989	1.00	47.44
	ATOM	124	CB	ASP A 20	23.471	9.355	44.187	1.00	60.22
40	ATOM	125	CG	ASP A 20	23.633	10.169	45.437	1.00	73.16

	ATOM	126	OD1 ASP A 20	24.132	11.311	45.326	1.00	79.05
	ATOM	127	OD2 ASP A 20	23.275	9.675	46.524	1.00	78.87
	ATOM	128	C ASP A 20	24.843	8.946	42.149	1.00	37.42
	ATOM	129	O ASP A 20	25.954	8.521	42.472	1.00	38.02
5	ATOM	130	N ARG A 21	24.213	8.484	41.084	1.00	26.27
	ATOM	131	CA ARG A 21	24.883	7.495	40.267	1.00	33.90
	ATOM	132	CB ARG A 21	23.930	6.931	39.223	1.00	36.79
	ATOM	133	CG ARG A 21	22.513	7.382	39.490	1.00	50.60
	ATOM	134	CD ARG A 21	21.547	6.260	39.495	1.00	46.09
10	ATOM	135	NE ARG A 21	21.068	5.971	38.157	1.00	44.95
	ATOM	136	CZ ARG A 21	20.533	6.865	37.329	1.00	42.44
	ATOM	137	NH1 ARG A 21	20.397	8.132	37.692	1.00	49.97
	ATOM	138	NH2 ARG A 21	20.103	6.474	36.138	1.00	34.61
	ATOM	139	C ARG A 21	25.951	8.301	39.565	1.00	36.17
15	ATOM	140	O ARG A 21	25.786	9.474	39.374	1.00	37.51
	ATOM	141	N GLY A 22	27.056	7.707	39.184	1.00	35.48
	ATOM	142	CA GLY A 22	27.983	8.551	38.437	1.00	28.95
	ATOM	143	C GLY A 22	27.566	8.445	36.983	1.00	24.58
	ATOM	144	O GLY A 22	26.409	8.046	36.674	1.00	22.09
20	ATOM	145	N PHE A 23	28.465	8.786	36.065	1.00	15.51
	ATOM	146	CA PHE A 23	28.157	8.704	34.664	1.00	15.18
	ATOM	147	CB PHE A 23	27.665	10.080	34.127	1.00	20.92
	ATOM	148	CG PHE A 23	28.539	11.242	34.571	1.00	20.47
	ATOM	149	CD1 PHE A 23	28.271	11.908	35.762	1.00	24.30
25	ATOM	150	CD2 PHE A 23	29.644	11.596	33.815	1.00	20.21
	ATOM	151	CE1 PHE A 23	29.126	12.965	36.237	1.00	25.52
	ATOM	152	CE2 PHE A 23	30.520	12.636	34.261	1.00	20.52
	ATOM	153	CZ PHE A 23	30.259	13.307	35.458	1.00	22.56
	ATOM	154	C PHE A 23	29.397	8.332	33.920	1.00	20.77
30	ATOM	155	O PHE A 23	30.484	8.463	34.494	1.00	24.08
	ATOM	156	N TYR A 24	29.242	7.891	32.673	1.00	20.54
	ATOM	157	CA TYR A 24	30.396	7.562	31.821	1.00	25.88
	ATOM	158	CB TYR A 24	30.393	6.126	31.295	1.00	33.47
	ATOM	159	CG TYR A 24	29.706	5.125	32.115	1.00	31.26
35	ATOM	160	CD1 TYR A 24	30.356	4.551	33.204	1.00	43.74
	ATOM	161	CE1 TYR A 24	29.763	3.573	33.939	1.00	47.84
	ATOM	162	CD2 TYR A 24	28.422	4.680	31.785	1.00	41.08
	ATOM	163	CE2 TYR A 24	27.818	3.692	32.524	1.00	39.08
	ATOM	164	CZ TYR A 24	28.492	3.151	33.589	1.00	44.15
40	ATOM	165	OH TYR A 24	27.949	2.164	34.357	1.00	56.07

	ATOM	166	C	TYR	A	24	30.332	8.362	30.588	1.00	24.71
	ATOM	167	O	TYR	A	24	29.264	8.859	30.221	1.00	31.49
	ATOM	168	N	PHE	A	25	31.473	8.437	29.901	1.00	21.41
	ATOM	169	CA	PHE	A	25	31.546	9.091	28.625	1.00	18.28
5	ATOM	170	CB	PHE	A	25	32.885	9.831	28.479	1.00	22.56
	ATOM	171	CG	PHE	A	25	32.945	11.076	29.294	1.00	22.04
	ATOM	172	CD1	PHE	A	25	33.336	11.040	30.625	1.00	22.70
	ATOM	173	CD2	PHE	A	25	32.496	12.281	28.757	1.00	29.30
	ATOM	174	CE1	PHE	A	25	33.264	12.190	31.432	1.00	25.64
10	ATOM	175	CE2	PHE	A	25	32.415	13.440	29.557	1.00	28.31
	ATOM	176	CZ	PHE	A	25	32.794	13.394	30.888	1.00	27.30
	ATOM	177	C	PHE	A	25	31.385	8.046	27.534	1.00	20.94
	ATOM	178	O	PHE	A	25	30.992	8.341	26.411	1.00	21.90
	ATOM	179	N	ASN	A	26	31.708	6.793	27.868	1.00	25.83
15	ATOM	180	CA	ASN	A	26	31.598	5.697	26.899	1.00	27.61
	ATOM	181	CB	ASN	A	26	33.020	5.296	26.396	1.00	28.31
	ATOM	182	CG	ASN	A	26	33.737	6.469	25.737	1.00	29.50
	ATOM	183	OD1	ASN	A	26	34.438	7.267	26.395	1.00	38.35
	ATOM	184	ND2	ASN	A	26	33.508	6.626	24.462	1.00	32.35
20	ATOM	185	C	ASN	A	26	30.924	4.534	27.633	1.00	23.53
	ATOM	186	O	ASN	A	26	31.274	4.214	28.753	1.00	23.60
	ATOM	187	N	LYS	A	27	29.958	3.879	27.025	1.00	26.93
	ATOM	188	CA	LYS	A	27	29.325	2.795	27.791	1.00	33.05
	ATOM	189	CB	LYS	A	27	28.018	2.384	27.113	1.00	34.53
25	ATOM	190	CG	LYS	A	27	27.014	3.521	27.084	1.00	37.05
	ATOM	191	CD	LYS	A	27	26.126	3.416	25.878	1.00	39.60
	ATOM	192	CE	LYS	A	27	24.768	4.078	26.139	1.00	42.35
	ATOM	193	NZ	LYS	A	27	24.244	4.674	24.865	1.00	39.82
	ATOM	194	C	LYS	A	27	30.242	1.603	27.870	1.00	31.68
30	ATOM	195	O	LYS	A	27	30.732	1.173	26.848	1.00	31.77
	ATOM	196	N	PRO	A	28	30.494	1.065	29.073	1.00	35.47
	ATOM	197	CD	PRO	A	28	30.001	1.491	30.392	1.00	34.96
	ATOM	198	CA	PRO	A	28	31.388	-0.110	29.148	1.00	36.66
	ATOM	199	CB	PRO	A	28	31.468	-0.435	30.637	1.00	40.26
35	ATOM	200	CG	PRO	A	28	30.367	0.359	31.287	1.00	40.80
	ATOM	201	C	PRO	A	28	30.784	-1.252	28.322	1.00	36.18
	ATOM	202	O	PRO	A	28	29.558	-1.344	28.152	1.00	36.79
	ATOM	203	N	THR	A	29	31.614	-2.107	27.763	1.00	34.63
	ATOM	204	CA	THR	A	29	31.026	-3.155	26.945	1.00	41.92
40	ATOM	205	CB	THR	A	29	31.824	-3.391	25.661	1.00	51.94

	ATOM	206	OG1 THR A 29	32.938	-4.249	25.947	1.00	52.79
	ATOM	207	CG2 THR A 29	32.313	-2.071	25.086	1.00	55.75
	ATOM	208	C THR A 29	30.897	-4.498	27.635	1.00	35.36
	ATOM	209	O THR A 29	30.068	-5.322	27.238	1.00	34.58
5	ATOM	210	N GLY A 30	31.721	-4.720	28.643	1.00	33.36
	ATOM	211	CA GLY A 30	31.659	-5.997	29.323	1.00	33.97
	ATOM	212	C GLY A 30	32.109	-7.243	28.536	1.00	31.86
	ATOM	213	O GLY A 30	32.227	-7.275	27.305	1.00	34.94
	ATOM	214	N TYR A 31	32.265	-8.325	29.275	1.00	23.05
10	ATOM	215	CA TYR A 31	32.723	-9.576	28.690	1.00	25.72
	ATOM	216	CB TYR A 31	33.144	-10.505	29.813	1.00	21.15
	ATOM	217	CG TYR A 31	34.274	-9.992	30.633	1.00	24.03
	ATOM	218	CD1 TYR A 31	34.066	-9.497	31.892	1.00	17.01
	ATOM	219	CE1 TYR A 31	35.106	-8.997	32.644	1.00	26.09
15	ATOM	220	CD2 TYR A 31	35.579	-9.983	30.121	1.00	29.13
	ATOM	221	CE2 TYR A 31	36.616	-9.502	30.870	1.00	25.81
	ATOM	222	CZ TYR A 31	36.383	-9.009	32.115	1.00	25.77
	ATOM	223	OH TYR A 31	37.419	-8.560	32.875	1.00	34.26
	ATOM	224	C TYR A 31	31.678	-10.274	27.840	1.00	29.45
20	ATOM	225	O TYR A 31	30.468	-10.172	28.112	1.00	30.08
	ATOM	226	N GLY A 32	32.141	-10.990	26.808	1.00	29.62
	ATOM	227	CA GLY A 32	31.228	-11.746	25.972	1.00	33.28
	ATOM	228	C GLY A 32	30.235	-10.912	25.198	1.00	36.13
	ATOM	229	O GLY A 32	29.161	-11.380	24.832	1.00	32.23
25	ATOM	230	N SER A 33	30.606	-9.668	24.937	1.00	41.08
	ATOM	231	CA SER A 33	29.722	-8.787	24.217	1.00	47.10
	ATOM	232	CB SER A 33	30.130	-7.331	24.386	1.00	46.73
	ATOM	233	OG SER A 33	29.397	-6.557	23.463	1.00	52.64
	ATOM	234	C SER A 33	29.816	-9.142	22.764	1.00	57.93
30	ATOM	235	O SER A 33	30.807	-9.735	22.317	1.00	57.01
	ATOM	236	N SER A 34	28.772	-8.755	22.039	1.00	65.04
	ATOM	237	CA SER A 34	28.657	-8.989	20.613	1.00	70.41
	ATOM	238	CB SER A 34	28.414	-7.659	19.899	1.00	72.65
	ATOM	239	OG SER A 34	27.049	-7.299	19.995	1.00	72.85
35	ATOM	240	C SER A 34	29.885	-9.671	20.028	1.00	71.58
	ATOM	241	O SER A 34	30.642	-9.053	19.289	1.00	69.77
	ATOM	242	CB THR A 41	30.810	6.812	19.043	1.00	59.11
	ATOM	243	OG1 THR A 41	29.666	5.952	18.975	1.00	64.04
	ATOM	244	CG2 THR A 41	31.511	6.892	17.700	1.00	59.18
40	ATOM	245	C THR A 41	31.044	6.416	21.449	1.00	51.54

	ATOM	246	O	THR	A	41	30.689	5.415	22.079	1.00	55.44
	ATOM	247	N	THR	A	41	32.206	4.887	19.817	1.00	58.60
	ATOM	248	CA	THR	A	41	31.763	6.289	20.105	1.00	57.60
	ATOM	249	N	GLY	A	42	30.804	7.654	21.870	1.00	35.08
5	ATOM	250	CA	GLY	A	42	30.159	7.853	23.151	1.00	21.75
	ATOM	251	C	GLY	A	42	29.409	9.184	23.225	1.00	18.36
	ATOM	252	O	GLY	A	42	29.011	9.724	22.200	1.00	21.32
	ATOM	253	N	ILE	A	43	29.298	9.708	24.431	1.00	21.07
	ATOM	254	CA	ILE	A	43	28.475	10.899	24.619	1.00	22.17
10	ATOM	255	CB	ILE	A	43	28.247	11.113	26.148	1.00	18.01
	ATOM	256	CG2	ILE	A	43	29.474	11.764	26.819	1.00	20.05
	ATOM	257	CG1	ILE	A	43	27.124	12.102	26.413	1.00	20.02
	ATOM	258	CD1	ILE	A	43	26.793	12.170	27.882	1.00	22.13
	ATOM	259	C	ILE	A	43	28.947	12.145	23.921	1.00	25.61
15	ATOM	260	O	ILE	A	43	28.118	12.955	23.466	1.00	18.04
	ATOM	261	N	VAL	A	44	30.260	12.336	23.748	1.00	19.35
	ATOM	262	CA	VAL	A	44	30.629	13.560	23.090	1.00	19.83
	ATOM	263	CB	VAL	A	44	32.187	13.809	23.157	1.00	22.51
	ATOM	264	CG1	VAL	A	44	32.587	14.916	22.238	1.00	29.90
20	ATOM	265	CG2	VAL	A	44	32.568	14.056	24.605	1.00	22.31
	ATOM	266	C	VAL	A	44	30.137	13.492	21.655	1.00	18.37
	ATOM	267	O	VAL	A	44	29.664	14.477	21.127	1.00	20.08
	ATOM	268	N	ASP	A	45	30.257	12.308	21.027	1.00	19.78
	ATOM	269	CA	ASP	A	45	29.821	12.142	19.657	1.00	22.36
25	ATOM	270	CB	ASP	A	45	30.132	10.738	19.207	1.00	29.90
	ATOM	271	CG	ASP	A	45	31.588	10.395	19.435	1.00	38.30
	ATOM	272	OD1	ASP	A	45	32.374	10.700	18.516	1.00	34.84
	ATOM	273	OD2	ASP	A	45	31.929	9.881	20.546	1.00	39.04
	ATOM	274	C	ASP	A	45	28.304	12.345	19.531	1.00	23.03
30	ATOM	275	O	ASP	A	45	27.830	13.023	18.613	1.00	22.16
	ATOM	276	N	GLU	A	46	27.610	11.772	20.489	1.00	21.13
	ATOM	277	CA	GLU	A	46	26.139	11.784	20.505	1.00	24.96
	ATOM	278	CB	GLU	A	46	25.676	10.660	21.418	1.00	32.84
	ATOM	279	CG	GLU	A	46	24.220	10.314	21.307	1.00	38.09
35	ATOM	280	CD	GLU	A	46	23.877	8.973	21.913	1.00	41.51
	ATOM	281	OE1	GLU	A	46	24.713	8.378	22.621	1.00	41.76
	ATOM	282	OE2	GLU	A	46	22.739	8.505	21.689	1.00	45.60
	ATOM	283	C	GLU	A	46	25.472	13.084	20.952	1.00	24.92
	ATOM	284	O	GLU	A	46	24.450	13.450	20.376	1.00	23.35
40	ATOM	285	N	CYS	A	47	26.045	13.747	21.970	1.00	20.20

	ATOM	286	CA	CYS A 47	25.477	14.961	22.590	1.00	19.56
	ATOM	287	C	CYS A 47	26.174	16.282	22.474	1.00	28.23
	ATOM	288	O	CYS A 47	25.563	17.328	22.769	1.00	21.99
	ATOM	289	CB	CYS A 47	25.240	14.687	24.091	1.00	21.60
5	ATOM	290	SG	CYS A 47	24.245	13.186	24.373	1.00	26.79
	ATOM	291	N	CYS A 48	27.452	16.263	22.074	1.00	18.45
	ATOM	292	CA	CYS A 48	28.181	17.537	21.940	1.00	17.21
	ATOM	293	C	CYS A 48	28.382	17.818	20.473	1.00	19.88
	ATOM	294	O	CYS A 48	28.082	18.890	20.040	1.00	23.54
10	ATOM	295	CB	CYS A 48	29.521	17.456	22.686	1.00	22.56
	ATOM	296	SG	CYS A 48	30.678	18.814	22.279	1.00	25.89
	ATOM	297	N	PHE A 49	28.854	16.830	19.706	1.00	19.44
	ATOM	298	CA	PHE A 49	29.044	17.010	18.279	1.00	26.47
	ATOM	299	CB	PHE A 49	29.939	15.912	17.710	1.00	27.72
15	ATOM	300	CG	PHE A 49	31.343	15.940	18.268	1.00	32.07
	ATOM	301	CD1	PHE A 49	32.098	14.764	18.363	1.00	31.76
	ATOM	302	CD2	PHE A 49	31.902	17.133	18.703	1.00	29.85
	ATOM	303	CE1	PHE A 49	33.396	14.798	18.889	1.00	32.64
	ATOM	304	CE2	PHE A 49	33.203	17.153	19.230	1.00	29.98
20	ATOM	305	CZ	PHE A 49	33.938	15.987	19.320	1.00	24.87
	ATOM	306	C	PHE A 49	27.706	17.000	17.573	1.00	27.42
	ATOM	307	O	PHE A 49	27.561	17.598	16.517	1.00	31.83
	ATOM	308	N	ARG A 50	26.744	16.298	18.167	1.00	29.59
	ATOM	309	CA	ARG A 50	25.381	16.219	17.640	1.00	26.05
25	ATOM	310	CB	ARG A 50	25.106	14.832	17.157	1.00	23.82
	ATOM	311	CG	ARG A 50	25.916	14.488	15.899	1.00	26.06
	ATOM	312	CD	ARG A 50	25.953	13.031	15.742	1.00	29.51
	ATOM	313	NE	ARG A 50	26.583	12.750	14.479	1.00	39.53
	ATOM	314	CZ	ARG A 50	27.849	12.386	14.370	1.00	41.06
30	ATOM	315	NH1	ARG A 50	28.603	12.260	15.466	1.00	40.37
	ATOM	316	NH2	ARG A 50	28.354	12.198	13.163	1.00	40.98
	ATOM	317	C	ARG A 50	24.434	16.539	18.794	1.00	25.89
	ATOM	318	O	ARG A 50	24.864	16.563	19.966	1.00	19.42
	ATOM	319	N	SER A 51	23.161	16.811	18.501	1.00	25.83
35	ATOM	320	CA	SER A 51	22.229	17.080	19.633	1.00	28.49
	ATOM	321	CB	SER A 51	21.122	18.082	19.248	1.00	29.44
	ATOM	322	OG	SER A 51	20.679	17.787	17.954	1.00	42.62
	ATOM	323	C	SER A 51	21.569	15.761	20.025	1.00	22.34
	ATOM	324	O	SER A 51	21.229	14.963	19.164	1.00	28.05
40	ATOM	325	N	CYS A 52	21.385	15.517	21.325	1.00	21.96

	ATOM	326	CA	CYS A 52	20.771	14.278	21.762	1.00	24.73
	ATOM	327	C	CYS A 52	19.637	14.683	22.708	1.00	25.51
	ATOM	328	O	CYS A 52	19.616	15.780	23.240	1.00	23.80
	ATOM	329	CB	CYS A 52	21.791	13.373	22.494	1.00	24.55
5	ATOM	330	SG	CYS A 52	22.331	13.922	24.151	1.00	28.05
	ATOM	331	N	ASP A 53	18.671	13.815	22.896	1.00	27.35
	ATOM	332	CA	ASP A 53	17.622	14.228	23.789	1.00	28.62
	ATOM	333	CB	ASP A 53	16.248	13.872	23.201	1.00	34.84
	ATOM	334	CG	ASP A 53	16.078	12.408	22.968	1.00	39.80
10	ATOM	335	OD1	ASP A 53	16.598	11.616	23.782	1.00	44.65
	ATOM	336	OD2	ASP A 53	15.413	12.052	21.965	1.00	46.35
	ATOM	337	C	ASP A 53	17.848	13.616	25.147	1.00	23.60
	ATOM	338	O	ASP A 53	18.735	12.762	25.324	1.00	21.40
	ATOM	339	N	LEU A 54	17.012	14.005	26.099	1.00	20.38
15	ATOM	340	CA	LEU A 54	17.163	13.558	27.477	1.00	14.25
	ATOM	341	CB	LEU A 54	16.015	14.169	28.329	1.00	21.53
	ATOM	342	CG	LEU A 54	16.024	13.807	29.805	1.00	22.22
	ATOM	343	CD1	LEU A 54	17.283	14.383	30.476	1.00	21.85
	ATOM	344	CD2	LEU A 54	14.733	14.336	30.497	1.00	20.36
20	ATOM	345	C	LEU A 54	17.276	12.046	27.683	1.00	23.79
	ATOM	346	O	LEU A 54	18.075	11.598	28.496	1.00	23.74
	ATOM	347	N	ARG A 55	16.449	11.253	26.978	1.00	23.24
	ATOM	348	CA	ARG A 55	16.489	9.795	27.104	1.00	26.67
	ATOM	349	CB	ARG A 55	15.469	9.140	26.140	1.00	33.74
25	ATOM	350	CG	ARG A 55	14.013	9.234	26.618	1.00	47.08
	ATOM	351	CD	ARG A 55	13.838	8.500	27.974	1.00	59.43
	ATOM	352	NE	ARG A 55	13.580	9.398	29.104	1.00	68.64
	ATOM	353	CZ	ARG A 55	14.359	9.527	30.182	1.00	73.73
	ATOM	354	NH1	ARG A 55	15.485	8.813	30.302	1.00	74.93
30	ATOM	355	NH2	ARG A 55	14.028	10.401	31.134	1.00	72.23
	ATOM	356	C	ARG A 55	17.876	9.229	26.774	1.00	25.72
	ATOM	357	O	ARG A 55	18.365	8.348	27.470	1.00	28.77
	ATOM	358	N	ARG A 56	18.482	9.765	25.726	1.00	21.19
	ATOM	359	CA	ARG A 56	19.785	9.285	25.276	1.00	27.70
35	ATOM	360	CB	ARG A 56	20.049	9.798	23.892	1.00	35.27
	ATOM	361	CG	ARG A 56	19.140	9.134	22.892	1.00	42.72
	ATOM	362	CD	ARG A 56	19.352	7.624	22.897	1.00	52.32
	ATOM	363	NE	ARG A 56	20.565	7.280	22.150	1.00	59.61
	ATOM	364	CZ	ARG A 56	21.142	6.075	22.108	1.00	61.66
40	ATOM	365	NH1	ARG A 56	20.646	5.046	22.789	1.00	64.42

	ATOM	366	NH2 ARG A 56	22.244	5.906	21.389	1.00	62.77
	ATOM	367	C ARG A 56	20.850	9.724	26.246	1.00	25.14
	ATOM	368	O ARG A 56	21.755	8.952	26.618	1.00	25.99
	ATOM	369	N LEU A 57	20.743	10.967	26.689	1.00	21.87
5	ATOM	370	CA LEU A 57	21.684	11.448	27.701	1.00	22.79
	ATOM	371	CB LEU A 57	21.367	12.923	28.059	1.00	22.01
	ATOM	372	CG LEU A 57	22.188	13.715	29.089	1.00	22.86
	ATOM	373	CD1 LEU A 57	21.537	15.099	29.190	1.00	27.83
	ATOM	374	CD2 LEU A 57	22.115	13.120	30.453	1.00	32.64
10	ATOM	375	C LEU A 57	21.665	10.578	28.970	1.00	20.53
	ATOM	376	O LEU A 57	22.720	10.208	29.525	1.00	19.79
	ATOM	377	N GLU A 58	20.486	10.199	29.479	1.00	18.07
	ATOM	378	CA GLU A 58	20.425	9.390	30.682	1.00	17.97
	ATOM	379	CB GLU A 58	18.951	9.351	31.198	1.00	24.34
15	ATOM	380	CG GLU A 58	18.883	8.941	32.660	1.00	36.31
	ATOM	381	CD GLU A 58	17.473	9.016	33.227	1.00	41.86
	ATOM	382	OE1 GLU A 58	17.316	8.902	34.474	1.00	39.29
	ATOM	383	OE2 GLU A 58	16.549	9.198	32.406	1.00	36.76
	ATOM	384	C GLU A 58	21.005	7.942	30.537	1.00	14.19
20	ATOM	385	O GLU A 58	21.331	7.279	31.508	1.00	26.60
	ATOM	386	N MET A 59	21.172	7.487	29.307	1.00	15.83
	ATOM	387	CA MET A 59	21.767	6.166	29.115	1.00	21.59
	ATOM	388	CB MET A 59	21.626	5.746	27.672	1.00	23.71
	ATOM	389	CG MET A 59	20.195	5.145	27.372	1.00	27.46
25	ATOM	390	SD MET A 59	19.916	5.067	25.648	1.00	38.20
	ATOM	391	CE MET A 59	18.000	5.126	25.597	1.00	38.66
	ATOM	392	C MET A 59	23.261	6.169	29.521	1.00	23.34
	ATOM	393	O MET A 59	23.859	5.124	29.663	1.00	25.38
	ATOM	394	N TYR A 60	23.847	7.353	29.726	1.00	19.16
30	ATOM	395	CA TYR A 60	25.266	7.386	30.144	1.00	18.17
	ATOM	396	CB TYR A 60	25.982	8.549	29.452	1.00	18.20
	ATOM	397	CG TYR A 60	26.144	8.364	27.992	1.00	20.05
	ATOM	398	CD1 TYR A 60	25.193	8.855	27.086	1.00	20.40
	ATOM	399	CE1 TYR A 60	25.339	8.675	25.713	1.00	22.99
35	ATOM	400	CD2 TYR A 60	27.245	7.686	27.482	1.00	23.58
	ATOM	401	CE2 TYR A 60	27.398	7.501	26.131	1.00	24.88
	ATOM	402	CZ TYR A 60	26.475	7.980	25.258	1.00	25.27
	ATOM	403	OH TYR A 60	26.676	7.780	23.940	1.00	24.52
	ATOM	404	C TYR A 60	25.406	7.424	31.634	1.00	22.93
40	ATOM	405	O TYR A 60	26.519	7.507	32.199	1.00	22.51



	ATOM	406	N	CYS A	61	24.290	7.385	32.352	1.00	18.42
	ATOM	407	CA	CYS A	61	24.402	7.303	33.809	1.00	15.63
	ATOM	408	C	CYS A	61	24.808	5.872	34.207	1.00	19.70
	ATOM	409	O	CYS A	61	24.394	4.908	33.563	1.00	25.04
5	ATOM	410	CB	CYS A	61	23.065	7.562	34.511	1.00	22.45
	ATOM	411	SG	CYS A	61	22.415	9.181	34.212	1.00	24.27
	ATOM	412	N	ALA A	62	25.543	5.738	35.298	1.00	24.74
	ATOM	413	CA	ALA A	62	26.004	4.424	35.756	1.00	31.14
	ATOM	414	CB	ALA A	62	27.273	4.588	36.641	1.00	24.85
10	ATOM	415	C	ALA A	62	24.902	3.747	36.563	1.00	36.05
	ATOM	416	O	ALA A	62	23.920	4.394	36.962	1.00	33.02
	ATOM	417	N	PRO A	63	25.014	2.427	36.780	1.00	44.06
	ATOM	418	CD	PRO A	63	26.021	1.447	36.310	1.00	47.05
	ATOM	419	CA	PRO A	63	23.942	1.803	37.576	1.00	46.88
15	ATOM	420	CB	PRO A	63	24.182	0.296	37.393	1.00	47.68
	ATOM	421	CG	PRO A	63	25.651	0.166	37.068	1.00	49.88
	ATOM	422	C	PRO A	63	24.111	2.253	39.027	1.00	48.13
	ATOM	423	O	PRO A	63	23.135	2.412	39.773	1.00	54.36
	ATOM	424	N	LEU A	64	25.379	2.467	39.379	1.00	51.56
20	ATOM	425	CA	LEU A	64	25.835	2.896	40.693	1.00	61.37
	ATOM	426	CB	LEU A	64	26.997	1.994	41.170	1.00	63.79
	ATOM	427	CG	LEU A	64	26.761	0.984	42.312	1.00	60.23
	ATOM	428	CD1	LEU A	64	26.872	-0.435	41.767	1.00	61.06
	ATOM	429	CD2	LEU A	64	27.781	1.210	43.447	1.00	58.43
25	ATOM	430	C	LEU A	64	26.327	4.343	40.607	1.00	67.33
	ATOM	431	O	LEU A	64	27.441	4.612	40.132	1.00	71.54
	ATOM	432	C1	CYC B	1	19.808	21.082	25.297	1.00	29.47
	ATOM	433	C6	CYC B	1	19.974	19.730	24.564	1.00	31.45
	ATOM	434	O21	CYC B	1	21.316	19.561	24.000	1.00	30.05
30	ATOM	435	C5	CYC B	1	19.707	18.570	25.545	1.00	24.70
	ATOM	436	C4	CYC B	1	18.349	18.647	26.185	1.00	28.97
	ATOM	437	C11	CYC B	1	18.069	17.503	27.203	1.00	25.55
	ATOM	438	C10	CYC B	1	18.915	17.625	28.449	1.00	26.59
	ATOM	439	C9	CYC B	1	18.774	19.062	29.167	1.00	22.28
35	ATOM	440	C15	CYC B	1	19.668	19.202	30.407	1.00	18.50
	ATOM	441	C18	CYC B	1	19.488	18.173	31.529	1.00	17.96
	ATOM	442	C17	CYC B	1	20.033	18.861	32.738	1.00	20.63
	ATOM	443	C16	CYC B	1	20.478	20.370	32.210	1.00	19.19
	ATOM	444	C43	CYC B	1	20.408	21.271	33.357	1.00	17.91
40	ATOM	445	C55	CYC B	1	21.476	20.723	34.438	1.00	26.47

	ATOM	446	C56	CYC	B	1	21.498	21.442	35.738	1.00	31.90
	ATOM	447	C57	CYC	B	1	22.417	22.653	36.015	1.00	39.26
	ATOM	448	N59	CYC	B	1	22.473	23.332	37.133	1.00	46.97
	ATOM	449	C74	CYC	B	1	23.443	24.507	37.216	1.00	56.76
5	ATOM	450	C75	CYC	B	1	22.897	25.835	36.400	1.00	62.01
	ATOM	451	C76	CYC	B	1	23.920	27.038	36.528	1.00	66.74
	ATOM	452	N77	CYC	B	1	23.715	27.349	37.968	1.00	68.00
	ATOM	453	C78	CYC	B	1	24.623	27.135	39.017	1.00	72.42
	ATOM	454	C80	CYC	B	1	24.134	27.596	40.383	1.00	76.46
10	ATOM	455	O86	CYC	B	1	22.731	28.037	40.272	1.00	73.03
	ATOM	456	C81	CYC	B	1	25.194	28.755	40.862	1.00	85.35
	ATOM	457	O87	CYC	B	1	25.451	29.858	39.876	1.00	86.03
	ATOM	458	C82	CYC	B	1	24.767	29.384	42.284	1.00	92.20
	ATOM	459	O88	CYC	B	1	23.400	29.939	42.115	1.00	92.22
15	ATOM	460	C83	CYC	B	1	25.777	30.529	42.728	1.00	96.59
	ATOM	461	O89	CYC	B	1	27.124	29.924	42.873	1.00	99.38
	ATOM	462	C84	CYC	B	1	25.395	31.205	44.130	1.00	97.03
	ATOM	463	O85	CYC	B	1	26.318	32.274	44.541	1.00	95.57
	ATOM	464	O79	CYC	B	1	25.765	26.665	38.880	1.00	74.76
20	ATOM	465	C68	CYC	B	1	21.737	23.120	38.324	1.00	39.41
	ATOM	466	C69	CYC	B	1	20.223	23.496	38.137	1.00	38.89
	ATOM	467	C70	CYC	B	1	19.523	23.355	39.421	1.00	38.22
	ATOM	468	N71	CYC	B	1	20.564	22.987	40.353	1.00	50.02
	ATOM	469	C72	CYC	B	1	20.399	22.763	41.607	1.00	55.62
25	ATOM	470	C90	CYC	B	1	21.157	21.536	42.121	1.00	57.83
	ATOM	471	O96	CYC	B	1	20.407	20.319	41.993	1.00	56.55
	ATOM	472	C91	CYC	B	1	21.664	21.940	43.578	1.00	63.18
	ATOM	473	O97	CYC	B	1	21.600	20.757	44.471	1.00	63.88
	ATOM	474	C92	CYC	B	1	23.186	22.499	43.622	1.00	66.58
30	ATOM	475	O98	CYC	B	1	23.971	21.462	44.307	1.00	68.12
	ATOM	476	C93	CYC	B	1	23.135	23.867	44.446	1.00	67.49
	ATOM	477	O99	CYC	B	1	22.357	24.774	43.597	1.00	66.74
	ATOM	478	C94	CYC	B	1	24.493	24.656	44.683	1.00	74.63
	ATOM	479	O95	CYC	B	1	24.178	25.865	45.490	1.00	80.12
35	ATOM	480	O73	CYC	B	1	19.735	23.526	42.349	1.00	63.35
	ATOM	481	O58	CYC	B	1	23.106	22.977	35.125	1.00	40.42
	ATOM	482	C54	CYC	B	1	20.742	22.893	32.977	1.00	18.54
	ATOM	483	C14	CYC	B	1	19.521	20.648	31.087	1.00	23.61
	ATOM	484	C19	CYC	B	1	17.911	20.963	31.644	1.00	16.19
40	ATOM	485	C13	CYC	B	1	19.810	21.781	30.035	1.00	19.97

	ATOM	486	O2O CYC B	1	21.223	21.655	29.535	1.00	19.41
	ATOM	487	C12 CYC B	1	18.975	21.668	28.869	1.00	17.80
	ATOM	488	C8 CYC B	1	19.095	20.197	28.184	1.00	20.86
	ATOM	489	C3 CYC B	1	18.129	20.072	26.867	1.00	26.70
5	ATOM	490	C7 CYC B	1	16.492	20.310	27.375	1.00	27.90
	ATOM	491	C2 CYC B	1	18.448	21.212	25.824	1.00	28.19
	ATOM	492	BR BR C	1	34.062	6.612	30.395	0.50	33.08
	ATOM	493	O HOH W	1	19.167	24.790	26.224	1.00	26.47
	ATOM	494	O HOH W	2	23.897	21.958	31.132	1.00	22.60
10	ATOM	495	O HOH W	3	21.553	23.642	27.485	1.00	29.42
	ATOM	496	O HOH W	4	24.680	19.970	37.794	1.00	27.43
	ATOM	497	O HOH W	5	30.918	19.401	40.676	1.00	23.25
	ATOM	498	O HOH W	6	16.708	17.200	40.896	1.00	28.59
	ATOM	499	O HOH W	7	16.516	25.080	41.353	1.00	25.88
15	ATOM	500	O HOH W	8	18.741	11.412	20.841	1.00	29.70
	ATOM	501	O HOH W	9	27.307	23.459	25.693	1.00	22.59
	ATOM	502	O HOH W	10	29.313	-4.298	41.568	1.00	37.18
	ATOM	503	O HOH W	11	24.485	23.989	32.854	1.00	35.58
	ATOM	504	O HOH W	12	17.430	11.457	36.970	1.00	26.36
20	ATOM	505	O HOH W	13	22.518	16.744	15.421	1.00	37.65
	ATOM	506	O HOH W	14	22.764	17.648	22.807	1.00	29.54
	ATOM	507	O HOH W	15	22.916	12.486	18.554	1.00	30.85
	ATOM	508	O HOH W	16	21.127	13.160	16.233	1.00	67.05
	ATOM	509	O HOH W	18	33.941	3.332	34.867	1.00	47.13
25	ATOM	510	O HOH W	19	28.659	24.132	37.361	1.00	34.15
	ATOM	511	O HOH W	20	26.049	12.100	38.687	1.00	43.38
	ATOM	512	O HOH W	22	22.998	5.484	43.119	1.00	56.07
	ATOM	513	O HOH W	23	21.091	10.590	19.804	1.00	37.65
	ATOM	514	O HOH W	24	27.553	20.681	37.920	1.00	40.51
30	ATOM	515	O HOH W	25	21.539	4.646	32.538	1.00	35.17
	ATOM	516	O HOH W	35	36.200	4.986	34.099	1.00	55.94
	ATOM	517	O HOH W	36	25.301	25.430	41.805	1.00	45.43
	ATOM	518	O HOH W	37	28.063	14.145	40.069	1.00	39.11
	ATOM	519	O HOH W	38	20.561	25.768	50.428	1.00	38.92
35	ATOM	520	O HOH W	39	22.841	23.744	25.080	1.00	40.88
	ATOM	521	O HOH W	40	16.781	11.726	39.998	1.00	65.62
	ATOM	522	O HOH W	41	29.705	23.094	40.234	1.00	40.48
	ATOM	523	O HOH W	42	18.375	14.116	41.324	1.00	58.97
	ATOM	524	O HOH W	43	15.538	9.778	20.639	1.00	61.25
40	ATOM	525	O HOH W	44	34.144	2.745	20.168	1.00	48.45

	ATOM	526	O	HOH W	45	20.621	8.952	42.429	1.00	33.04
	ATOM	527	O	HOH W	46	17.087	6.329	28.850	1.00	32.70
	ATOM	528	O	HOH W	47	25.800	23.668	39.622	1.00	46.80
	ATOM	529	O	HOH W	48	16.978	27.199	25.066	1.00	44.77
5	ATOM	530	O	HOH W	49	22.764	7.118	24.736	1.00	38.06
	ATOM	531	O	HOH W	50	24.770	22.332	46.924	1.00	48.70
	ATOM	532	O	HOH W	51	21.688	25.678	41.327	1.00	52.53
	ATOM	533	O	HOH W	52	19.396	17.922	42.396	1.00	53.49
	ATOM	534	O	HOH W	53	21.375	18.431	46.997	1.00	57.91
10	ATOM	535	O	HOH W	54	19.736	20.961	17.193	1.00	46.33
	ATOM	536	O	HOH W	55	30.374	4.915	45.161	1.00	52.15
	ATOM	537	O	HOH W	56	18.588	26.408	48.436	1.00	44.58
	ATOM	538	O	HOH W	57	23.722	24.504	28.990	1.00	38.53